2016

Studying the effects of Ubisol-Q10 in animal models of Alzheimer's and Parkinson's disease

Krithika Muthukumaran
University of Windsor

Studying the effects of Ubisol-Q_{10} in animal models of Alzheimer's and Parkinson's disease

by

Krithika Muthukumaran

A Dissertation

Submitted to the Faculty of Graduate Studies

Through the Department of Chemistry and Biochemistry

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy at the

University of Windsor

Windsor, Ontario, Canada

2016

Studying the effects of Ubisol-Q_{10} in animal models of Alzheimer's and Parkinson's disease

By

Krithika Muthukumaran

APPROVED BY:

_____
Dr. Donald Kuhn, External Examiner
Wayne State University

_____
Dr. D. Higgs
Department of Biological Sciences

_____
Dr. P. Vacratsis
Department of Chemistry & Biochemistry

_____
Dr. B. Mutus
Department of Chemistry & Biochemistry

_____
Dr. S. Pandey, Advisor
Department of Chemistry & Biochemistry

September, 6 2016

Declaration of co-authorship/previous publication

I. Declaration of Co-authorship

I hereby declare that this thesis incorporates materials that are a result of joint research. This thesis includes work carried out in collaboration with Dr. Jerome Cohen, Dr. Jagdeep Sandhu and Dr. Marianna Sikorska, under the supervision of Dr. Siyaram Pandey.

The collaboration with Dr. Cohen is covered in chapters 2-4.

The collaboration with Dr. Sandhu is covered in chapters 2, 3.

The collaboration with Dr. Sikorska is covered in chapters 2-4.

Contributions from the authors were covered in the acknowledgements sections of the published work, where appropriate.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis and the research to which it signifies, is the product of my own work.

II. Declaration of previous publications

Thesis chapter	Publication title/Full citation	Publication status
Chapter 2	Orally delivered water soluble Coenzyme Q_{10} (Ubisol-Q_{10}) blocks on-going neurodegeneration in rats exposed to paraquat: Potential for therapeutic application in Parkinson's disease. BMC Neurosci 14, 21.	Published
Chapter 3	Genetic susceptibility model of Parkinson's Disease Resulting from Exposure of DJ-1 deficient mice to MPTP: Evaluation of neuroprotection by Ubisol-Q_{10}. Journal of Parkinson's disease 4(3): 523 – 30.	Published
Chapter 4	Inhibition of amyloid plaque formation and memory deficits by Ubisol-Q_{10} (nanomicelle formulation of CoQ_{10}) in a double transgenic mouse model of Alzheimer's disease	In-progress

I certify that I have obtained a written permission from the copyright owner(s) to include the above published material(s) in my thesis. I certify that the above material describes work completed during my registration as graduate student at the University of Windsor.

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis.

I declare that this is a true copy of my thesis and that this thesis has not been submitted for a higher degree to any other University or Institution.

Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) commonly affect people 60 years and above. Since the current treatment options only provide symptomatic relief and there is no effective way to halt the disease progression, there is a potential global socioeconomic crisis. Both PD and AD could be caused due to genetic disposition or environmental factors; however, oxidative stress has been implicated as a major contributor to the pathophysiology of the disease. Hence, an ideal way to halt the disease progression would be to use an antioxidant which can scavenge the reactive oxygen species and protect the neurons.

In this study Ubisol–Q_{10}, a water soluble formulation of the antioxidant Coenzyme Q_{10} (CoQ_{10}) was tested on animal models of PD and AD. Ubisol–Q_{10} alleviated symptoms and halted loss of dopaminergic neurons when administered therapeutically to an environmental toxin paraquat rat model of PD, a model that mimics slow progressive neurodegeneration seen in PD. In the presence of Ubisol-Q_{10} treatment there was 16% loss of neurons in comparison to the PQ untreated group that suffered a significant 41% loss. Ubisol-Q_{10} also provides neuroprotection when administered prophylactically in a genetically susceptible DJ-1 deficient transgenic mouse model exposed to the neurotoxin 1-methyl-4-phenyl-1,2,3,6 acetic acid. In the MPTP injected DJ-1 mice there was a significant 49% loss in the number of dopaminergic neurons in comparison to the group that received prophylactic treatment group with Ubisol-Q_{10} which showed 19% loss of neurons. However, a short-term study conducted suggests that prolonged treatment with Ubisol-Q_{10} is necessary to sustain the neuroprotection

In vitro studies with Ubisol-Q_{10} reverse premature senescence in AD fibroblasts. Prophylactic studies were conducted with Ubisol-Q_{10} where it was administered over a 14 month period to a

transgenic mouse model of AD containing human amyloid precursor protein and a mutant presenilin 1. Treatment showed improved long term memory in the Y-maze and was accompanied with decrease in the levels of plaques in brain sections, reduced circulating human amyloid-beta and altered glial cells morphology in comparison to the untreated group.

The bioavailability of Ubisol-Q_{10} is high at small doses, 10 times lower than the FDA approved dose and the neuroprotection properties hold promise in providing effective treatment to those suffering from these diseases.

Acknowledgements

First, I would like to thank my supervisor Dr. Siyaram Pandey for giving me this wonderful opportunity and project. Thank you for your guidance and support this past five and a half years, which has helped me greatly to both learn more about neuroscience and neurodegenerative diseases and teach many undergraduate students. Your enthusiasm and positive outlook is very inspiring.

Thanks to my committee members Dr. Higgs, Dr. Mutus and Dr. Vacratsis for all the help and inputs to improve and better present my thesis. Thanks to Dr. Zielinski, Dr. Ananvoranich and Dr. Mutus for allowing me to use their lab equipment.

Thanks to our collaborator Dr. Cohen and his students, especially Marisa Vennettelli, Daniel Lopatin, Nicholas Guilbeault and Kaitlyn Iannicello, for conducting the behaviour studies, which helped to add a whole new dimension to the project. A special thanks to the animal care technicians Linda Sterling and Melissa Gabrieau, two of the most hard working people I know, for taking care of the animals, helping me with new techniques, when needed and for your friendship.

Thank you to Mr Joseph Scecsei and his family for supporting this project and for the love and encouragement.

Thanks to Dr. Arumugam Muruganandam for his continued support, guidance and encouragement, it is greatly appreciated. I am so thankful to Dr. Zhang for patiently answering my questions regarding neuroscience, job applications and helping me to believe in myself.

One of the best parts of this project was the opportunity to work with a number of undergraduates from Behaviour Cognition and Neuroscience program, who were all so passionate about neuroscience. I got the opportunity to work with an enthusiastic group of young

neuroscientists who helped me with the injections, dissections, sectioning, immunohistochemistry, and microscopy and with standardising protocols with new antibodies. I would like to thank Jessica Smith, Kate Harrison, Samantha Leahy, Harshil Jasra, Miranda Mazza, Joseph Correa, Melissa Macksoud, Alexandra Marginean, Austin Elliott, Annie Kanwar, and Simon Pupulin for their contributions to the project. Thanks to Dr. Alyson Laframboise for teaching me how to use the cryostat. You were so kind, supportive, a great teacher and always had this infectious enthusiasm for neuroscience.

So much gratitude to the department secretaries, Marlene Bezaire, Cathy Wilson and Beth Kickham for the unconditional love. I can't put to words how much you have done for me and thanks for always looking out for me. Thanks for making me a better person.

Thanks to all the past and present Pandey lab members who have been great colleagues and for all the help.

Thanks to the friends that I have made Matthew Steckle, Manika Gupta, Danny Tarade, Grace Teskey, Melissa Macksoud, Kyle Stokes, Jessica Smith, Melissa Cowell, Austin Elliott, Annie Kanwar and Alexandra Marginean for all the fun times and for standing by me when life got rough. This past five and half years would not have been a memorable journey without my dear friend Pamela Ovadje. I am so grateful for all your help. Especially when I broke my collar bone and had a surgery you were there for me and took such good care of me. Thanks for teaching me how to be so selfless and generous. I have had great times attending conferences with you and we have been there for each other during our ups and downs, I am sure this friendship will last a lifetime.

Thanks to Alexandra Marginean, Pamela Ovadje and Matthew Steckle, my mini editing committee for proofreading my thesis and for your valuable comments.

I would like to thank my parents for allowing me to pursue my higher studies and for all the love and support. I am aware that it has not been easy for you both to face questions day in and day out from relatives and friends about letting their only girl child to pursue higher education. I am so grateful and lucky to have parents who have been very encouraging and open minded.

A big thank you to Ms. Giselle St Louis as I would not have come this far in life without your words of wisdom.

Contents

Abbreviations

6-ODHA – 6-hydroxydopamine

Aβ – Amyloid beta

ABC – Avidin biotin complex

AD – Alzheimer's disease

ADH – Aldehyde dehydrogenase

AKT – Protein kinase B

ANOVA – Analysis of variance

AβPP – Amyloid precursor protein

ApoE4 – Apolipoprotein E4

BBB – Blood brain barrier

CA1 – Stratum pyramidale

CoQ_{10} – Coenzyme Q10

CSF – Cerebrospinal fluid

CuSOD – Cu Superoxide dismutase

DAT – Dopamine transporter

DBS – Deep brain stimulation

DOPAC – 3, 4 dihydroxyphenyl acetic acid

ETC – Electron transport chain

FDA – Food and drug administration

GABA – γ – aminobutyric acid

GDNF – Glial cells derived neurotrophic factor

GFAP – Glial fibrillary antigen

GLP – Good lab practices

GPe – Globus pallidus externus

GPi – Globus pallidus internus

GRAS – Generally regarded as safe

GSK3 – Glycogen synthase kinase 3

GSK - 3β – Glycogen synthase kinase 3 beta

L-DOPA – Levodopa

LRRK2 – Leucine rich repeat kinase 2

MAO – Monoamine oxidase

MAO B – Monoamine oxidase B

MAPT – Microtubule-associated protein tau

MnSOD – Mn Superoxide dismutase

MPP+ - 1-methyl-4-phenyl pyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6 acetic acid

NADPH-cytochrome P450 – CYP450

NFTs – Neurofibrillary tangles

NL – Novel location

NMDA – N-methyl-D-aspartic acid

NOR – Novel object location

NRC – National Research Centre

NSAID – Non-steroidal anti-inflammatory drugs

PBS – Phosphate buffered saline

PCD – Programmed cell death

PCNA – Proliferating cellular nuclear antigen

PD – Parkinson’s disease

PINK1 – PTEN-induced putative kinase

PQ – Paraquat

PS1 – Presenilin – 1

PS2 – Presenilin – 2

PTS – Polyoxyethanyl-α-tocopherol sebacate

ROS – Reactive oxygen species

SNCA – Synuclein A

SNpc – Substantia nigra pars compacta

TBS – Tris buffered saline

TH – Tyrosine hydroxylase

UPS – Ubiquitin proteasome system

WHO - World Health Organization

ZnSOD – Zn Superoxide dismutase

Chapter 1

General Introduction

Improved health care in developed countries has led to an increase in average lifespan. Thus, with an increasing ageing population in the developed countries, World Health Organization (WHO) predicts that by 2040, there will be a steep increase in the number of people affected by neurodegenerative diseases such as Alzheimer's disease (AD) and other causes of dementia, as well as diseases +that primarily affect the motor functioning such as Parkinson's disease (PD). This will result in neurodegenerative diseases overtaking cancer as the second leading cause of death after cardiovascular diseases. The treatment costs for neurodegenerative diseases currently accounts for $20 billion USD but will likely have a sharp increase in the coming decades, following the projected increase of neurodegenerative diseases [1]. The symptoms of these diseases cause disability and dependence of the older population thereby taking a toll on the caregivers and the family as well. The possible societal economic burden posed by AD and PD in the coming decades has led to an increase in investments and research to identify biomarkers for early detection and to find effective treatment options.

1.1 Parkinson's disease:

Parkinson's disease is a slow progressive neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the brain leading to motor symptoms such as resting tremors, postural instability, and rigidity. It is also accompanied with widespread neuronal changes resulting in variable and complex non-motor symptom [2]. It is commonly idiopathic and by the time the symptoms occur almost 60-70% of the dopaminergic neurons in the SNpc are already lost. One of the hallmarks of PD is the presence of Lewy bodies, which are cellular inclusions containing aggregates of α-synuclein and ubiquitin. Approximately

10 million people live with PD worldwide (www.pdf.org). Age is considered to be one of the main risk factors for PD and it has been estimated that about 5% of those diagnosed with PD are less than 60 years old (Reeve, Simcox, & Turnbull, 2014 [3].

1.1.1 Basal ganglia pathway:

Once the key pathological feature of PD was identified as selective loss of dopaminergic neurons in the SNpc region of the brain, the association was established between the basal ganglia circuit and PD. Dopamine is a neurotransmitter synthesized by the dopaminergic neurons and it has a regulatory role in the basal ganglia circuit which is involved in motor control. The basal ganglia circuit is complex and it is important to understand this circuit in detail in order to recognise the role played by dopamine in the circuit and the magnitude of the loss of dopaminergic neurons.

From specific cortical areas there are excitatory glutamatergic projections or inputs to selected areas in the striatum, namely the caudate putamen, caudate nucleus, and ventral striatum. The discharge from these striatal regions to the basal ganglia output nuclei is spontaneous. The basal ganglia output nuclei includes the globus pallidus internus (GPi), globus pallidus externus (GPe) and subthalamic nuclei. The output nuclei GPi in turn has a γ-aminobutyric acid (GABA) - mediated inhibitory effect on their target regions in the thalamus (Figure 1). The extent of inhibitory effect that the GPi has on the thalamus is modulated by two parallel and opposing pathways [4].

In the direct pathway there are inhibitory striatal efferent neurons containing GABA that project directly to the output nuclei GPi. Therefore, increased inhibition of GPi in the direct pathway results in disinhibition of the thalamus (Figure 1) [4].

In the indirect pathway, there are inhibitory GABA striatal projections to the GPe, followed by inhibitory GABA projections from the GPe to the subthalamic nucleus and finally excitatory

glutamate projections from the subthalamic nucleus to the GPi. An increase in spontaneous discharge from the GPe results in an increased inhibitory effect on the subthalamic nucleus. Hence, activation of the inhibitory striatal GABA projections to GPe is required for the subthalamic nucleus to activate GPi such that there is an increased inhibitory effect on the target nuclei in the thalamus (Figure 1) [4,5].

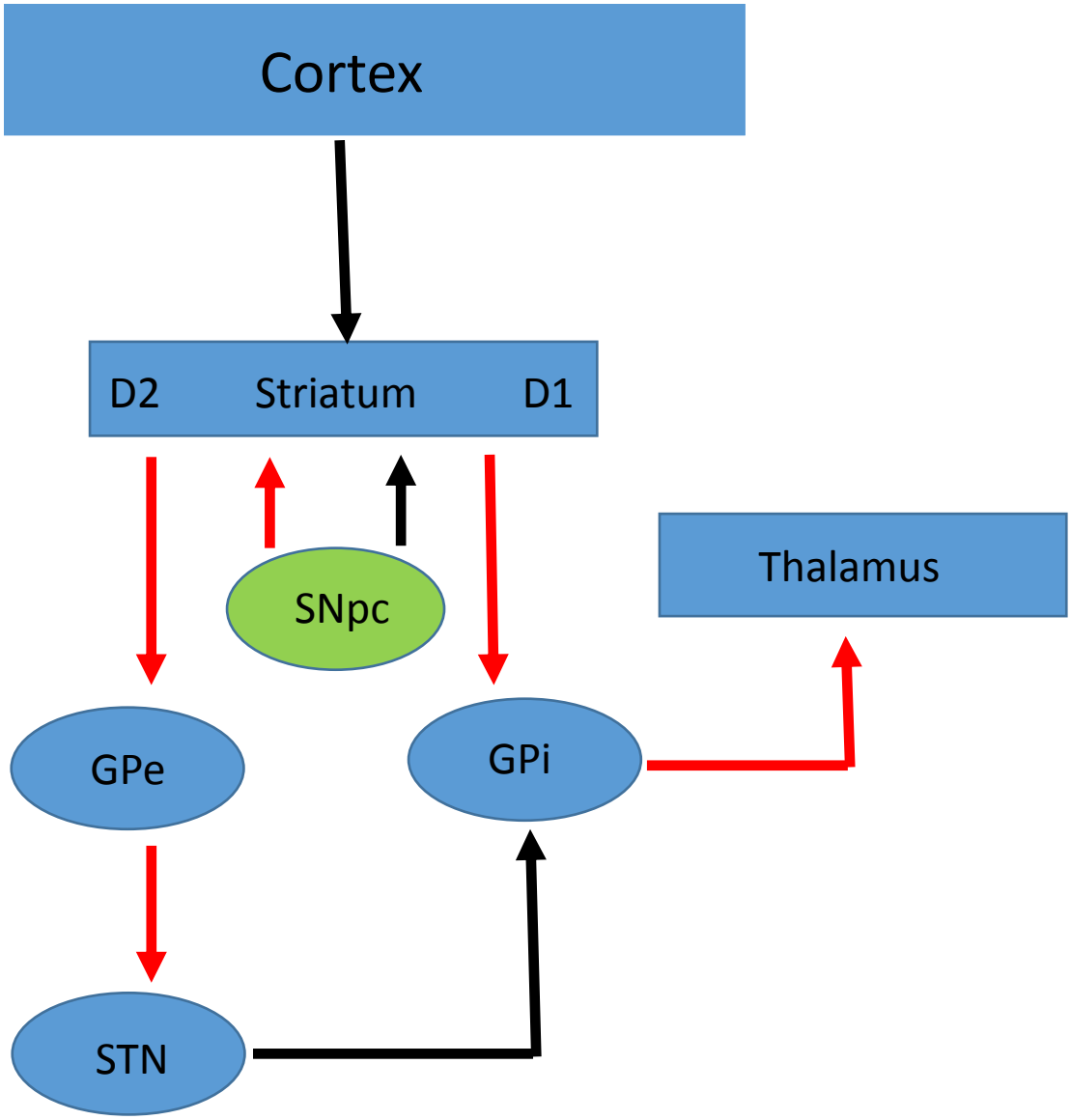


Figure 1. Basal ganglia circuitry. The parallel opposing pathways, direct and indirect pathways regulated by dopaminergic input from SNpc. Red arrows represent GABA inhibitory projections to the target neurons and the black arrows represent the glutamatergic excitatory projections to the target neurons. (SNpc – Substantia nigra pars compacta; GPe – Globus pallidus externus; GPi – Globus pallidus internus; STN – Subthalamic nucleus)

Dopamine, a neurotransmitter, has a complex role in the basal ganglia circuit with contrasting effects in the direct and indirect pathways. It has an excitatory effect on the striatal neurons that are part of the direct pathway and an inhibitory effect on the striatal neurons that take part in the indirect pathway. Dopamine helps reinforce the input obtained from the cortex to the basal ganglia thalamocortical circuit, thereby having a net excitatory or inhibitory effect on the thalamus [6]. The output projections from the thalamus that completes the circuit is not restricted to the primary motor cortex and is extended to different regions of the frontal lobe as well. In the case of PD, the dopaminergic neurons are lost thereby affecting the regulatory role played by dopamine in the basal ganglia circuit, leading to motor deficits.

1.1.2 Dopamine synthesis, metabolism and oxidative stress:

L-tyrosine is the amino acid required for dopamine biosynthesis and is present in abundance in dietary proteins. Circulating tyrosine reaches the brain via low affinity amino acid transporters, following which, they are specifically taken up by the dopaminergic neurons [7]. The first step in dopamine synthesis is the conversion of L-tyrosine to L-dihydroxy phenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH), which acts as the rate-limiting step. Aromatic amino acid decarboxylase then converts L-DOPA to dopamine [8]. The synthesized dopamine is stored in vesicles and the concentration of dopamine is 10 to 1000 times higher in the vesicles than in the cytoplasm of dopaminergic neurons [9].

An action potential in the dopaminergic neurons causes membrane depolarization that results in calcium uptake and fusion of the vesicles containing dopamine with the neuronal membrane. This is followed by exocytosis, releasing the vesicles with dopamine into the synaptic cleft [10].

An increased action potential in the dopaminergic neurons is accompanied with increase in the release of dopamine at two sites, dendrites and neuron terminal. There are two types of dopamine receptors on the striatal neurons, D1 and D2. D1 acts by increasing the activity of adenylate cyclase and is predominantly found on the striatal neurons involved in the direct pathway while D2 inhibits adenylate cyclase activity, and is present on the striatal neurons that participate in the indirect pathway [11].

The transporters present in the dopaminergic neurons play an important role in the maintenance of transmitter homeostasis. These high affinity membrane carriers transport dopamine in both directions depending on the concentration gradient, thereby enabling the recycling and uptake of the released dopamine back into the nerve terminal of dopaminergic neurons, where it can be metabolized [9]. The D2 type auto-receptors present on the dopaminergic neurons are activated by released dopamine and will in turn inhibit dopamine synthesis and release. This acts as a feedback mechanism to regulate the levels of dopamine in the synaptic cleft [12].

Metabolism of dopamine varies according to the abundance and activity of enzyme, cell type, region of the brain and species examined. The enzymes monoamine oxidase (MAO) and aldehyde dehydrogenase (ADH) are involved in the production of the metabolite 3, 4 dihydroxyphenyl acetic acid (DOPAC). This metabolite, DOPAC, is present both extra and intra-neuronally, with respect to the dopamine terminal and at significantly higher levels in the brains of rats [9]. The other common metabolite homovanillic acid (HVA) is present extra-neuronally, due to the presence of an additional enzyme, catechol-O-methyltransferase (COMT), and HVA is the metabolite commonly observed in the brains of humans and primates.

Dopamine has an important role in the nigrostriatal pathway, but it can also be neurotoxic, making the dopaminergic neurons highly vulnerable. Along with DOPAC and HVA, the

metabolism of dopamine via the MAO pathway also results in the production of H_2O_2. The increase in cytosolic H_2O_2 could result in the generation of hydroxyl radicals and hence lead to increased oxidative stress and vulnerability of dopaminergic neurons [13]. Dopamine is highly reactive and can undergo autoxidation thereby resulting in the generation of superoxide and H_2O_2. It can also react with cysteine residues in proteins and form cysteinyl-dopamine or cysteinyl-DOPAC conjugates [14].

1.1.3 PD animal model:

It is now accepted that PD is caused by a combination of both genetic and environmental factors. In the early 1980s, a group of young drug addicts were diagnosed with a subacute onset of severe Parkinsonism and it was later found that this was caused due to the presence of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) in the synthetic heroin analogues they consumed [15]. MPTP is lipophilic, allowing it to easily cross the blood brain barrier (BBB), where it is converted to its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) by the enzyme monoamine oxidase B (MAO B), an enzyme that participates in catecholamine degradation [16]. MPP+ is then specifically taken up by the dopaminergic neurons via the dopamine transporters (DAT) for which it has a high affinity [17]. In the cells, the concentration of MPP+ increases in the mitochondria where it blocks the complex I of the electron transport chain (ETC), thus decreasing energy production and increasing the levels of reactive oxygen species (ROS) [18,19]. It has been shown that transgenic mice overexpressing Cu/Zn superoxide dismutase (CuSOD/ZnSOD) and Mn superoxide dismutase (MnSOD) are able to significantly reduce the toxic effects posed by exposure to MPTP [20,21]. In mice and primates, MPTP exposure causes selective loss of dopaminergic neurons in the SNpc region of the brain, leading to motor deficits, similar to what is seen in patients suffering from PD [22,23]. However, PD is a slow progressive

disease that takes many years to develop, and in the MPTP mouse model, it is an acute or subacute process. Chronic administration of different doses of MPTP only resulted in uneven striatal dopamine loss [24].

A number of studies indicate that apoptosis and inflammation are the two important aspects that could be attributed to the selective neuronal loss observed with acute MPTP administration. There is increased DNA fragmentation, activation of caspase 3 and expression of Bax in the dopaminergic neurons of mice exposed to MPTP [25,26,27]. Also, mice overexpressing Bcl-2 or deficient in p53 or Bax have significant neuroprotection when exposed to MPTP [28,29,26]. The cell death process could be activated via cJUN kinase, as neuroprotection is observed in the presence of cJUN kinase inhibitors [30]. When the neuropathological changes in three patients exposed to MPTP for a period of 3-16 years was studied, clustering of reactive microglia at the vicinity of ongoing dopaminergic neuronal loss was seen [15]. There is both activated microglia and lymphocytes in the MPTP animal model as well, suggesting the presence of a similar Parkinsonian inflammatory response [31].

Even though MPTP is able to induce PD in animal models, only drug addicts and chemists are exposed to this toxin. It was later found that paraquat (PQ), an herbicide, has structural similarity to MPTP. Epidemiological studies conducted in California and Taiwan further confirmed that exposure to non-specific general pesticides and specific pesticides, such as PQ and rotenone, are associated with increased incidence of PD [32,33,34]. The two specific pesticides, PQ and rotenone, cause an increase in ROS; PQ induces redox cycling and rotenone blocks complex I of the ETC, thereby leading to an increase in ROS. However, in animal models of rotenone there is variable loss of dopaminergic neurons, therefore, they could not be used as a model to study the disease pathogenesis or test the various therapeutic options for PD [35].

1.1.4 Paraquat redox cycling:

PQ – 1, 1' dimethyl 4, 4'bipyridylium dichloride is a quaternary ammonium bipyridyl compound, a broad spectrum herbicide and exists as a divalent cation PQ^{2+} in its native state. In the presence of NADPH, PQ^{2+} is reduced to a monovalent cation PQ^{+} and NADPH is oxidized to NADP, by the enzyme NADPH oxidase. The reduction of PQ^{2+} is also accompanied by the generation of H_2O_2, $O_2^{\cdot-}$, and hydroxyl radicals, a strong oxidant. This redox cycling, where the two main consequences are depletion of NADPH and generation of ROS, contributes to the mechanism by which PQ induces cytotoxicity. It has been shown that the presence of oxygen and aerobic conditions are essential for PQ toxicity in mammals, plants and microorganisms [36]. An increase in the levels of PQ leads to the hijack of the pentose phosphate pathway by PQ, so as to increase the turnover of NADPH, which can stimulate the PQ redox cycle, causing oxidative stress and eventually cell death [37].

In the case of PD, there is a selective loss of dopaminergic neurons, hence, the possible mechanism of selectivity was evaluated. The reduced form, PQ^{+}, rather than PQ^{2+}, is the substrate for DAT, which selectively transports PQ^{+} to the dopaminergic neurons, where it initiates redox cycling [38]. The reduction of PQ^{2+} to PQ^{+} is carried out by the enzyme NADPH oxidase or other reducing agents in the microglia prior to its transport to the dopaminergic neurons. Studies have also shown that there is a dose dependent increase in the levels of NADPH oxidase expression in the dopaminergic neurons, an enzyme that is essential for the redox cycling to take place [39]. Use of inhibitors that block NADPH oxidase activity reduces the toxic effects of PQ, thereby suggesting the important role played by this enzyme in PQ induced cytotoxicity via redox cycling [39]. Therefore, differences in susceptibility to PQ across the population could be attributed to the differences in the active role played by NADPH oxidase in

the redox cycling and the natural antioxidant defense mechanisms that efficiently counteract the effects of ROS.

Toxicokinetic studies have been conducted on humans with acute PQ poisoning in order to understand the half-life and duration of PQ in the system. The results indicate that PQ can be recovered for several weeks or months following PQ poisoning and is released at a slow rate from the cells. This was confirmed by the parallel kinetic evolution of PQ in plasma, urine and cerebrospinal fluid samples [40]. PQ detoxification in mouse liver requires the activity of NADPH-cytochrome P450 (CYP450) and the survival rate of mice receiving 50 mg/Kg dose of PQ increases greatly when the mice are also treated with drugs that induce the activity of CYP450 [41]. Different isoforms of CYP450 have been identified and the link between the various isoforms and degrees of cytotoxicity suggests that CYP450 could also play a role in differential susceptibility to PD on exposure to PQ [42]. For example, subjects with CYP4502D6 polymorphism are poor metabolizers of pesticides resulting in a two- to six-fold increased risk to PD on exposure to pesticides [43].

Redox cycling of PQ and increased ROS generation is followed by an increase in the expression of Bax and oligomerization of Bax and Bak, leading to opening up of the mitochondrial apoptosis-induced channels and release of pro-apoptotic factors such as Smac and Diablo. This is followed by opening of the mitochondrial permeability transition pore, collapse of the mitochondrial membrane potential and the induction of programmed cell death (PCD). The mechanism of cytotoxicity for MPP+ and PQ could vary, however, they share an oxidative component [44].

1.1.5 Genetic model of PD:

Familial aggregation of PD has been widely studied and in the last decade, there are several genes that have been identified that have a clear link to familial cases of PD and follow Mendelian forms of the disease. These include autosomal dominant genes such as Synuclein A (SNCA), and Leucine rich repeat kinase 2 (LRRK2), while PTEN – induced putative kinase (PINK1), PARK2, and DJ-1 which are recessive. Among these genes, one of the commonly used animal model for familial cases of PD is the DJ-1 knockout transgenic mouse model [45].

1.1.5.1 DJ-1 and PD:

A mutation in the DJ-1 gene, which causes loss of function, is associated with autosomal recessive early-onset PD and is responsible for 1-2% of early onset familial cases of PD [46]. DJ-1 is a highly conserved protein that has a 189 amino acid sequence and is expressed at higher levels in the astrocytes compared to neurons [47]. This suggests that DJ-1 has a crucial role in glial cell biology and could be the possible link between DJ-1 mutations and neurodegeneration. DJ-1 acts as an antioxidant, where under oxidative stress, it undergoes self-oxidation, converting the cysteine 106 residue to cysteine sulfonic acid [48]. This ability of DJ-1 to undergo auto-oxidation enables it to act as a ROS scavenger [49]. Over-expression of DJ-1 *in vitro* aids in the prevention and elimination of any toxic injury caused by H_2O_2 [49]. The antioxidant property of DJ-1 may be attributed to it stabilising a master regulator of antioxidant transcriptional responses, Nrf2 and additionally preventing the association of Nrf2 to Keap1. When Nrf2 is bound to Keap1, it is unable to activate the transcription of a number of antioxidants and phase II detoxifying genes. DJ-1 also acts as a redox dependent chaperon and prevents α-synuclein aggregation and increases the levels of glutathione [50,51]. *In vitro* studies indicate a possible link between DJ-1 and mitochondrial functioning as DJ-1 deficiency leads to decrease in

mitochondrial membrane polarity, increase in mitochondrial fragmentation, and decreased calcium buffering by the mitochondria. Increased oxidative stress leads to translocation of DJ-1 to the mitochondria and it helps protect the cells from oxidative stress induced PCD [52,53,54].

The DJ-1 knockout transgenic mouse models do not exhibit loss of dopaminergic neurons, which is characteristic of PD but exhibit increased re-uptake of dopamine, thereby causing changes to striatal dopaminergic function and behavioral deficits [55]. However, the DJ-1 knockout mice have shown elevated vulnerability to MPTP and loss of dopaminergic neurons, which could be due to the absence of the antioxidant properties normally exhibited by DJ-1. We can conclude from these results that DJ-1 plays a critical role in the maintenance and survival of dopaminergic neurons and further studies on the DJ-1 animal model could aid in a better understanding of the pathogenesis of PD and act as a suitable model for the development of successful therapies for PD.

1.1.6 Therapeutic options - Current and future:

Once PD is diagnosed and the symptoms are evaluated, the ideal treatment approach is to customise therapy based on the needs of the patients. Levodopa (L-DOPA) is the most common drug, which can efficiently manage the symptoms that arise in PD, especially bradykinesia. In addition to L-DOPA, administration of carbidopa, an inhibitor of L-DOPA decarboxylase, aids in prolonging the effects of L-DOPA, thereby enhancing the therapeutic effects [56]. However, it has been shown that patients taking L-DOPA for more than 5 years begin to show motor fluctuations and dyskinesia. Anti- dyskinesia drugs such as clozapine, propranolol, amantadine, an N-methyl-D-aspartic acid (NMDA) receptor blocker, COMT and MAO inhibitors reduce the effects of prolonged treatment with L-DOPA, without having to decrease the dose of L-DOPA. In order to prevent these complications, one of the strategies is to delay the administration of L-

DOPA until the motor symptoms begin to affect the day-to-day activities [56]. Using dopamine agonists provides modest improvement, bypasses presynaptic neurons or dopamine synthesis and directly activates dopamine receptors, and therefore could be used to provide better motor control in the early stages of the disease. Patients primarily affected by tremors could be given anticholinergics and amantadine. Some of the novel anti-depressants that act on noradrenergic and serotonergic transmission have been shown to improve tremors and L-DOPA induced dyskinesia.

It has been shown that deprenyl, an antioxidant, is effective in preventing disease progression in an MPTP rodent model [57]. Similarly, there are antioxidants that act as substitutive electron carriers, such as Coenzyme Q10 (CoQ_{10}) and quinines. These compounds have been studied and clinical trials have been performed in order to examine their ability to halt disease progression and loss of dopaminergic neurons [58]. The majority of treatment options currently available only help improve PD symptoms, without addressing or saving the remaining dopaminergic neurons following diagnosis.

Furthermore, the interaction between dopamine and glutamate in the indirect pathway has been thought to play a role in the behavior symptoms that arise in PD. Hence blocking glutamate receptors by using NMDA receptor antagonists could be a method of alleviating the disease symptoms [59]. Furthermore, glial cell derived neurotrophic factor (GDNF) has been shown to prevent the loss of dopaminergic neurons and rescue the degenerating neurons *in vivo*.

A pilot study was carried out in PD diagnosed patients, whereby GDNF was delivered through a catheter inserted in the cerebro-ventricular region [60]. However, it showed very little improvement and the patients reported other side effects, including nausea, depression and hallucinations, leading to withdrawal of the clinical trial. In another study where GDNF was

infused to the putamen in 5 patients, there were no side effects reported and patients showed approximately 61% improvement in the daily activity sub score and reduced L-DOPA induced dyskinesia [61]. However, the clinical trials with GDNF had to come to a halt, when similar treatments with GDNF caused cerebral toxicity in primates.

Deep brain stimulation (DBS) is one of the most effective treatment options available for advanced stages of PD that targets GPi and subthalamic nuclei and brings about approximately 40% reduction in tremor. However, the stimulation required is continuous and it would be much more user friendly and efficient if the stimulation used a feedback control and was provided only when necessary. Pilot studies on primates and patients have been done where the stimulation was automized and provided based on the local field potential and accelerometer measurements of tremor activity. More trials need to be carried out and further fine-tuning of the optimum stimulation mechanism must be evaluated to manage the needs of the patients more effectively [62].

The option of stem cell therapy is being widely explored and evaluated in animal models, while the pros and cons of using different types of stem cells have been analyzed. However, more studies must be conducted before gaining FDA approval to have safe and successful clinical trials [63].

1.2 Alzheimer's disease:

Alois Alzheimer, a German psychiatrist first described the clinical features of AD based on his work with a woman who presented a peculiar form of dementia at the age of 51. A century later, AD still arouses curiosity and interest among neuroscientists as the disease pathology has yet to be fully deciphered and effective therapies are still lacking. There are currently 30 million patients suffering from AD worldwide and this number is expected to quadruple by 2050. This

establishes AD as the most important global public health issue. AD has no treatment options available to effectively halt or slowdown the disease progression and patients require an enormous amount of aid and support for extended periods of time [64].

At a macroscopic level, AD is characterized by gross atrophy of the brain. At the microscopic level, it is characterized by the presence of amyloid plaques in the extracellular space, neurofibrillary tangles (NFTs) which are intracellular structures, and extensive loss of synapses and neurons [65]. The amyloid plaque contains amyloid beta (Aβ) peptides that are 40 or 42 amino acids in length and are derived from the transmembrane protein, amyloid precursor protein (AβPP). AβPP is expressed at higher levels in the neurons and has three sites that can undergo proteolytic cleavage. Aβ is generated by the action of two different proteases: β-secretase and γ-secretase. A third protease, α-secretase generates a smaller fragment and no Aβ. The γ-secretase enzyme can cleave AβPP at multiple sites, thereby generating various isoforms of Aβ, out of which Aβ40 is found in abundance in the brain and cerebrospinal fluid (CSF). $A\beta_{1-42}$ is present at low concentrations, approximately 10% of the levels of $A\beta_{1-40}$, and tends to aggregate by forming fibrils and oligomers. The $A\beta_{1-42}$ aggregates are hydrophobic and insoluble, thereby establishing the dense core of the Aβ plaques after which the incorporation of $A\beta_{1-40}$ and non-Aβ components, such as ubiquitin and α-synuclein, will follow [66]. These plaques are surrounded by degenerating neurons and gliosis, which can be recognised by altered morphology and proliferation of astrocytes and microglia. There is evidence of chronic inflammation in the brain of people suffering from AD, leading to inflammatory response, activation of microglia at the vicinity of Aβ plaque, and neurotoxic effects [67]. Since inflammation and activation of microglia could contribute to the disease pathogenesis, non-

steroidal anti-inflammatory drugs (NSAIDs) are being tested as a treatment option for AD [68]. However, the status and role of astrocytes in AD pathogenesis is still unclear.

The NFTs present in an AD brain are hyperphosphorylated aggregates of tau, a protein present in both neurons and glial cells that binds to microtubules [69]. Tau, when bound to microtubules, aid in stabilizing the microtubules and regulates its structure and function. This binding is regulated by the phosphorylation status and other post-translational modifications of tau. However, when tau is hyperphosphorylated by glycogen synthase kinase 3 (GSK3), as in the case of AD, it dissociates from the microtubules, self-aggregates and forms NFTs within the neurons thereby leading to cytoskeletal instability [70]. There is an axonal gradient with higher concentration of tau closer to the synapse where it participates in synaptic activity by interacting with post-synaptic signalling complexes, regulates glutamatergic receptor content in the dendrites, and influences functioning of the synaptic mitochondria. Phosphorylation of certain tau residues can also regulate long-term depression and it is these synaptic modifications that form the basis for learning and memory [71]. Mutations in the tau encoding microtubule-associated protein tau (MAPT) gene could lead to familial cases of AD and currently thirty-five mutations in over one-hundred families have been identified. Missense mutations in the MAPT gene affects the binding of tau to microtubules whereas silent mutations alter the tau isoform ratio thereby leading to taupathies. Furthermore, in adults, there are six isoforms of tau produced, due to alternate splicing of mRNA. The different isoforms are localised in different regions in the developmental and adult brain, varying in functions. Identification of these tau mutations has led to the development of animal models that can successfully exhibit the molecular and cellular features of taupathies [72,73].

The last hallmark of AD is the loss of synapses and neurons in selected regions of the brain such as the layer II of etorhinal cortex, pyramidal layers like the CA1 region of the hippocampus, certain regions of the temporal parietal and frontal cortex [74]. The majority of neurons lost use glutamate as the neurotransmitter, along with some cholinergic and noradrenergic neurons. This observation has led to the use of drugs that inhibit cholinesterase, an enzyme that breaks down acetylcholine, and memantine, a drug that modulates one of the subtypes of NMDA receptors to manage the symptoms in early and moderate stages of AD [74]. The AβPP/P1SK1 is a transgenic mouse model that exhibits abundant CA1 neuronal loss accompanied by memory deficits and is preceded by intracellular accumulation of Aβ [75].

1.2.1. Amyloid plaque hypothesis:

The greatest challenge, as well as controversy, lies in understanding the sequence of events that leads to the observed morphological and biochemical changes in AD. The amyloid cascade has been studied and accepted for decades, leading to the development of multiple drugs that can reduce the plaque deposits. According to the amyloid plaque hypothesis, amyloid plaque deposition is the primary event, which is responsible for the disease progression and the pathogenesis of AD [76]. However, focusing on the amyloid cascade hypothesis alone has not addressed the various pathologies observed in this multi-faceted disease. Trials with a number of small molecules and immunotherapy that specifically targets Aβ plaques are underway, but have failed to efficiently prevent disease progression [77]. This has led to questioning the significance of amyloid plaque in AD pathogenesis.

The presence of neuronal cell death in the vicinity of amyloid plaques in the hippocampus and frontotemporal cortices suggests that Aβ does have neurotoxic effects [78,79]. Studies have also shown that Aβ produces an inflammatory response, and generates H_2O_2 and oxidative stress at

the vicinity of the plaque [80]. Similar lesions and inflammatory responses have been observed when primates were given intra-cortical injections of $A\beta_{1-42}$ [81].

The most valid evidence supporting the role of amyloid plaque in the disease progression came with the characterization of genes involved in familial cases of AD leading to early onset of AD. When there are mutations in the genes involved in Aβ generation, this leads to increased production and accumulations of Aβ. Mutations in the AβPP gene, which is located in chromosome 21, results in increased Aβ generation; there is a similar increase in Aβ accumulation observed in patients suffering from Down syndrome as they exhibit trisomy in chromosome 21 [82,83,84]

Mutations in presenilin-1 (PS1) and presenilin-2 (PS2), which are part of the catalytic site of γ-secretase enzyme, also lead to elevated levels of Aβ. Additionally, it has been found that the presence of the ApolipoproteinE4 (ApoE4) allele is responsible for late onset AD and is thought to play a role in disease pathogenesis by binding to Aβ with a high affinity, thus accelerating the formation of Aβ fibrils [85,86,87]. In transgenic mice that contain mutations in AβPP and PS genes, behavior deficits arise following plaque deposits; plaque deposition is the only AD pathology that can be mimicked by these double transgenic mice [88]. However, in triple transgenic mice that contain AβPP, PS and tau mutations, the Aβ plaque formation precedes extensive tau hyperphosphorylation and formation of NFTs, favoring the amyloid cascade hypothesis [89]. The accumulation of Aβ and NFT in AD clearly indicates deficits in the basal neuronal housekeeping functions. There is mounting evidence suggesting that in AD pathophysiology the autophagosome-lysosome mechanism is impaired and there is decreased expression of Beclin1, a protein involved in autophagy. Therefore, an imbalance between Aβ

generation and clearance, due to the failure in autophagy, may be a factor initiating the disease pathophysiology in an ageing population [90].

Immunohistochemistry studies have shown a four-fold increase in caspase cleaved AβPP in the hippocampus region of AD patients in comparison to age-matched controls where there was an inverse relationship with a decline in the levels of caspase cleaved AβPP with age. Caspase cleavage of AβPP is a normal physiological process with differences in the distribution of cleaved AβPPs. In the AD brain, there is more positive staining for caspase-cleaved APP seen in the cytoplasm and nuclei in the early stages of AD unlike in the non-AD cases [91]. In order to gain a better understanding of the sequence of events, transgenic mice expressing familial Swedish and Indiana AβPP mutations were created, with a point mutation at the caspase cleavage site. These transgenic mice did proceed to have Aβ deposits, but were protected from memory deficits, synaptic loss, and electrophysiological changes [92,93,94]. *In vitro* studies show that Aβ interactions with the extracellular domain of AβPP facilitates its oligomerization and this interaction and oligomerization is necessary for the subsequent cleavage of AβPP by caspases [95.96]. These results suggest that the physiological deficits exhibited in the AD transgenic mouse model occur downstream to Aβ plaque formation and caspase cleavage of AβPP is required for the observed AD symptoms.

Amyloid precursor protein could be a dependence receptor, where the absence of certain trophic factors may lead to the increased cleavage of AβPP by caspases and subsequently programmed cell death (PCD) [97]. For example, Netrin-1, which has multiple roles in axon guidance, is known to interact with both AβPP and Aβ, and the absence of Netrin-1 could lead to the neuronal cell death seen in AD. There are currently two schools of thought regarding this hypothesis. The first suggests that it is the increase in the levels of Aβ that blocks the binding of

Netrin-1 to AβPP, leading to activation of caspases and finally apoptosis [98]. On the other hand, the absence of trophic factors such as Netrin-1 is due to changes in the physiological signalling at the synapse, where the amyloidogenic pathway is favored, leading to Aβ accumulation and simultaneously, there is loss of synapse, caspase activation and PCD. These changes in the physiological signalling at the synapse could be attributed to decrease in energy production, increase in calcium influx, metabolic stress and glutamate excitotoxicity [99,100].

These observations and conclusions indicate that Aβ accumulation does have a role in the disease pathogenesis and neurodegeneration observed in AD. However, this may not be a primary event in AD pathogenesis since there are multiple changes and pathways that are simultaneously activated, together contributing to the disease pathogenesis. The loss of neurons is a late event, which is preceded by loss of synapses and neuritic retraction; thus, evidence demonstrating that changes in the physiological signalling pathways leads up to apoptosis.

One strong argument against the amyloid cascade hypothesis, where Aβ supposedly triggers AD pathogenesis, has been that the ageing process is normally accompanied by Aβ production without always having implications on cognition and memory, as seen in AD [101]. Similarly, although patients with Down Syndrome have increased levels of Aβ accumulation in the brain and the CSF, they do not all show signs of AD (http://www.alz.org/dementia/down-syndrome-alzheimers-symptoms.asp). Furthermore, majority of AD is sporadic, whereas early-onset AD, due to genetic mutations, encompasses just 1% of all AD diagnoses and hence mutations that result in Aβ accumulation may represent only one possible mechanism of the disease pathogenesis. A comprehensive understanding of the etiology of AD is still unknown and it is possible that there are multiple mechanisms and courses of action that take place. An increase in Aβ deposition has also been found in times of trauma and as a response to CNS injury [102,103].

This leads to the idea that Aβ might act as a compensation mechanism, where instead of having a toxic effect, Aβ acts as an antioxidant to enable the attenuation of metal induced oxidative damage. Hence, Aβ could be a bystander and a protector, rather than the cause of the disease [104,105].

As a result, an alternate way to approach AD treatment would be to address the metabolic and oxidative abnormalities, along with the toxic effects caused by these plaques. One solution that would address these conditions would be the use of an antioxidant that can efficiently quench the ROS and reduce PCD in order to curb the disease progression. Furthermore, using an antioxidant could prevent disease progression and the key pathological features associated with AD, if oxidative damage and mitochondrial dysfunction indeed precedes Aβ generation. With age, there is a decline in the levels of antioxidants in the brain, in addition to impaired mitochondrial bioenergetics transport and dynamics, which could also contribute greatly to the pathogenesis of AD. The role of the mitochondria in synapses with regards to calcium buffering at the synaptic terminals and aiding with energy requirements has been reported [106]. Therefore, using an antioxidant that can quench the ROS and help renew normal mitochondrial functioning may be an effective method to approach AD treatment.

1.2.2. Therapeutic options for AD:

With a better understanding of Aβ plaques, drugs targeting the two enzymes, β- and γ-secretases, were developed. However, in addition to AβPP, these enzymes have multiple substrates, hence, the drugs caused severe impairments and the clinical trials failed [107,108]. NMDA receptor antagonists have been approved for AD therapy. The excessive release of glutamate and over-stimulation of receptors that lead to increased calcium influx has been linked to the pathological hallmarks observed in AD. NMDA receptor antagonists have proven to be

effective in early and moderate stages of the disease [109]. Similarly, receptors of the neurotransmitters serotonin and acetylcholine have also been commonly tested to better manage the symptoms associated with AD. A poly-pharmacological approach could be most efficient, where multiple receptor targets are modified simultaneously [110]. Rasagiline is a MAO-B inhibitor that has been used in the treatment of PD. Currently derivatives of this inhibitor is being tested as a treatment option for AD as they have been shown to activate Bcl-2, downregulate Bax, and favor the cleavage of APP by α-secretase, thereby preventing the formation of Aβ plaques [111].

Insulin signalling is impaired in AD leading to an increased risk of Type 2 diabetes in AD patients. Therefore, the use of glucagon-like peptide-1 analogues and glucagon-like peptide receptor agonists, which promote insulin secretion have been shown to both reduce the Aβ load and improve cognition and synaptic plasticity [112,113].

Since oxidative stress, increase in ROS and mitochondrial dysfunction are thought to play a substantial role in ageing and in the disease pathogenesis of both PD and AD, the use of CoQ_{10} is theoretically an effective therapeutic. CoQ_{10} is a lipid soluble antioxidant, and a cofactor in the ETC. *In vitro,* CoQ_{10} is able to prevent PQ induced ROS generation, mitochondrial membrane depolarization, apoptosis and DNA fragmentation [114,115]. However, the ability of CoQ_{10} to prevent oxidative damage *in vivo* has not shown consistent results because of poor bioavailability, limiting clinical applications, although beneficial effects have been demonstrated. CoQ_{10} is essential for the functioning of all cell types but the tissue distribution of CoQ_{10} is variable, which may be due to differences in their functional consequences. The current testing of CoQ_{10} has been for clinical conditions where tissue damage is believed to be caused by oxidative stress and is followed by inflammatory response [116].

Our collaborators in National Research Council (NRC), Ottawa have developed a technique to solubilize CoQ_{10} and enable improved bioavailability and delivery of the lipophilic molecule. This is done by using α-tocopherol derivatized to polyoxyethanyl α-tocopherol sebacate (PTS). PTS can combine with CoQ_{10} in a 3:1 mol/mol ratio to form a non-covalent PTS:CoQ_{10} called Ubisol-Q_{10}. This formulation can remain stable and is created by heating PTS and CoQ_{10} at a temperature just above their melting points in order to form a melt, which is water-soluble [117,118]. CoQ_{10} is naturally part of the ETC and hence the role of Ubisol-Q_{10} in preventing the events in the mitochondria during apoptosis has been characterized. In the presence of oxidative stress, generated by H_2O_2 or other agents, there is an increase in the levels of expression of Bax accompanied by an increase in ROS and release of pro-apoptotic factors such as cytochrome c. However, pre-treatment with Ubisol-Q_{10} is able to prevent Bax induced collapse of mitochondrial membrane potential and cell death. Similar results were obtained when experiments were conducted with isolated mitochondria and recombinant Bax, where Ubisol-Q_{10} is able to prevent Bax induced mitochondrial collapse [119]. *In vitro*, in mixed cultures of neurons and astrocytes derived from human NT2/D1 cells, Ubisol-Q_{10} prevents glutamate excitotoxicity, which would normally cause increased ROS and ATP depletion [120]. Ubisol-Q_{10} inhibits stress induced premature senescence in AD fibroblasts obtained from patients with PS-1 mutations by decreasing the levels of ROS [121]. Prophylactic treatment with Ubisol-Q_{10} also helps in providing neuroprotection in an environmental toxin paraquat rat model of PD [122].

1.3 Objectives:

Advancements have been made to better understand the pathophysiology of AD and PD and a number of clinical trials are underway to identify better treatment options. However, we still lack a treatment option that can successfully halt the disease progression and the drugs currently in use only help provide symptomatic relief. Since *in vitro* studies have shown very encouraging results and prophylactic treatment with Ubisol-Q_{10} in a PQ rat model was able to prevent neurodegeneration it was best to expand the study further and understand the efficacy of Ubisol-Q_{10} as a treatment option for AD and PD. The following are the objectives for the study:

1) Do rats exposed to paraquat show slow progressive neurodegeneration?
2) Does this formulation cross the blood brain barrier?
3) Does Ubisol–Q_{10} have therapeutic effect in paraquat rat model of PD (immediate intervention)?
4) Is prolonged treatment with Ubisol – Q_{10} required to provide significant neuroprotection?
5) Does prophylactic treatment with Ubisol – Q_{10} provide neuroprotection in genetically susceptible DJ-1 mouse model exposed to the toxin MPTP
6) Does Ubisol – Q_{10} ameliorate the pathological and behavioural changes in a APP/PS1 transgenic mouse model of AD.

1.4 References:

1. Gammon, K. (2014). Neurodegenerative disease: Brain windfall. *Nature*. *515*, 299-300.
2. Mhyre, T. R., Boyd, J. T., Hamill, R. W., & Maguire-Zeiss, K. A. (2012). Parkinson's disease. *Sub-cellular biochemistry*, *65*: 389-455.
3. Reeve, A., Simcox, E., & Turnbull, D. (2014). Ageing and Parkinson's disease: Why is advancing age the biggest risk factor? *Ageing Research Reviews*, *14*(1), 19–30.
4. Kreitzer, A. C., & Malenka, R. C. (2008). Striatal Plasticity and Basal Ganglia Circuit Function. *Neuron*, *60*(4), 543–554.
5. Nelson, A. B., Kreitzer, A. C., Institutes, T. G., Francisco, S., Francisco, S., & Francisco, S. (2015). Reassessing Models of Basal Ganglia Function and Dysfunction. *Annu Rev Neurosci*, 1–18.
6. Calabresi, P., Picconi, B., Tozzi, A., Ghiglieri, V., & Di Filippo, M. (2014). Direct and indirect pathways of basal ganglia: a critical reappraisal. *Nature Neuroscience*, *17*(8), 1022–1030.
7. Kvetnansky, R., Sabban, E. L., & Palkovits, M. (2009). Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiological Reviews*, *89*(2), 535–606.
8. Best, J. A., Nijhout, H. F., & Reed, M. C. (2009). Homeostatic mechanisms in dopamine synthesis and release: a mathematical model. *Theoretical Biology and Medical Modelling*,
9. Eisenhofer, G. (2004). Catecholamine Metabolism: A Contemporary View with Implications for Physiology and Medicine. *Pharmacological Reviews*, *56*(3), 331–349.
10. Shin, O. H. (2014). Exocytosis and synaptic vesicle function. *Comprehensive Physiology*, *4*(1), 149–175.
11. Surmeier, D. J., Ding, J., Day, M., Wang, Z., & Shen, W. (2007). D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends in Neurosciences*, *30*(5), 228–235.

12. Ford, C. P. (2014). The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. *Neuroscience*, 12(282), 13-22.
13. Yamato, M., Kudo, W., Shiba, T., Yamada, K., Watanabe, T., & Utsumi, H. (2010). Determination of reactive oxygen species associated with the degeneration of dopaminergic neurons during dopamine metabolism. *Free Radical Research*, *44*(March), 249–257.
14. Chen, L., Ding, Y., Cagniard, B., Van Laar, A. D., Mortimer, A., Chi, W., … Zhuang, X. (2008). Unregulated Cytosolic Dopamine Causes Neurodegeneration Associated with Oxidative Stress in Mice. *Journal of Neuroscience*, *28*(2), 425–433.
15. Langston, J. W., Forno, L. S., Tetrud, J., Reeves, A. G., Kaplan, J. A., & Karluk, D. (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Annals of Neurology*, *46*(4), 598–605.
16. Tipton, K. F., & Singer, T. P. (1993). Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *Journal of Neurochemistry*, *61*(4), 1191–206.
17. Bezard, E., Gross, C. E., Fournier, M. C., Dovero, S., Bloch, B., & Jaber, M. (1999). Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp Neurol*, *155*(2), 268–273.
18. Chan, P., Delanney, L. E., Irwin, I., Langston, J. W., & Monte, D. Di. (1991). Tetrahydropyridine in Mouse Brain. *Journal of Neurochemistry*, 348–351.
19. Smeyne, R. J., & Jackson-Lewis, V. (2005). The MPTP model of Parkinson's disease. *Molecular Brain Research*, *134*(1), 57–66.
20. Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn, S., … Cadet, J. L. (1992). Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J Neurosci*, *12*(5), 1658–1667.
21. Klivenyi, P., St Clair, D., Wermer, M., Yen, H. C., Oberley, T., Yang, L., & Flint Beal, M. (1998). Manganese superoxide dismutase overexpression attenuates MPTP toxicity.

Neurobiology of Disease, *5*(4), 253–8.

22. Porras, G., Li, Q., & Bezard, E. (2012). Modeling Parkinson's disease in primates: The MPTP model. *Cold Spring Harbor Perspectives in Medicine*, *2*(3), 1–10.
23. Sikorska, M., Lanthier, P., Miller, H., Beyers, M., Sodja, C., Zurakowski, B., … Sandhu, J. K. (2014). Nanomicellar formulation of coenzyme Q10 (Ubisol-Q10) effectively blocks ongoing neurodegeneration in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model: Potential use as an adjuvant treatment in Parkinson's disease. *Neurobiology of Aging*, *35*(10), 2329–2346.
24. Hantraye, P., Varastet, M., Peschanski, M., Riche, D., Cesaro, P., C. Willer, J., & Maziere, M. (1993). Stable parkinsonian syndrome and uneven loss of striatal dopamine fibres following chronic MPTP administration in baboons. *Neuroscience*, *53*(1), 169–178.
25. Tatton, N. a, & Kish, S. J. (1997). In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience*, *77*(4), 1037–1048.
26. Hartmann, a, Hunot, S., Michel, P. P., Muriel, M. P., Vyas, S., Faucheux, B. a, … Hirsch, E. C. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(6), 2875–80.
27. Vila, M., Jackson-Lewis, V., Vukosavic, S., Djaldetti, R., Liberatore, G., Offen, D., … Przedborski, S. (2001). Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(5), 2837–2842.
28. Trimmer, P. A., Smith, T. S., Jung, A. B., & Bennett, J. P. (1996). Dopamine neurons from transgenic mice with a knockout of the p53 gene resist MPTP neurotoxicity. *Neurodegeneration*, *5*(3), 233–9.
29. Yang, L., Matthews, R. T., Schulz, J. B., Klockgether, T., Liao, a W., Martinou, J. C., … Beal,

M. F. (1998). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *18*(20), 8145–8152.

30. Saporito, M. S., Thomas, B. A., & Scott, R. W. (2000). MPTP activates c-Jun NH2-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. *Journal of Neurochemistry*, *75*(3), 1200–1208.
31. Kurkowska-jastrze, I., & Wron, A. (1999). The Inflammatory Reaction Following. *Experimental Neurology*, *156*, 50–61.
32. Liou, H. H., Tsai, M. C., Chen, C. J., Jeng, J. S., Chang, Y. C., Chen, S. Y., & Chen, R. C. (1997). Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. *Neurology*, *48*(6), 1583–1588.
33. Gatto, N. M., Cockburn, M., Bronstein, J., Manthripragada, A. D., & Ritz, B. (2009). Well-water consumption and Parkinson's disease in rural California. *Environmental Health Perspectives*, *117*(12), 1912–1918.
34. Berry, C., La Vecchia, C., & Nicotera, P. (2010). Paraquat and Parkinson's disease. *Cell Death and Differentiation*, *17*(7), 1115–25.
35. Beal
36. Bus, J. S., & Gibson, J. E. (1984). Paraquat: Model for oxidant-initiated toxicity. *Environmental Health Perspectives*, *55*, 37–46.
37. Lei, S., Zavala-Flores, L., Garcia-Garcia, A., Nandakumar, R., Huang, Y., Madayiputhiya, N., … Franco, R. (2014). Alterations in energy/redox metabolism induced by mitochondrial and environmental toxins: A specific role for glucose-6-phosphate-dehydrogenase and the pentose phosphate pathway in paraquat toxicity. *ACS Chemical Biology*, *9*(9), 2032–2048.
38. Rappold, P. M., Cui, M., Chesser, A. S., Tibbett, J., Grima, J. C., Duan, L., … Tieu, K. (2011). Paraquat neurotoxicity is mediated by the dopamine transporter and organic cation transporter-3. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(51),

20766–71.

39. Cristóvão, A. C., Choi, D.-H., Baltazar, G., Beal, M. F., & Kim, Y.-S. (2009). The role of NADPH oxidase 1-derived reactive oxygen species in paraquat-mediated dopaminergic cell death. *Antioxidants & Redox Signaling*, *11*(9), 2105–18.
40. Houz, P., & Scherrmann, J. M. (1990). Toxicokinetics of paraquat in human. *Human & Experimental Toxicology*, *9*, 5-12.
41. Shimada, H., Furuno, H., Hirai, K. I., Koyama, J., Ariyama, J., & Simamura, E. (2002). Paraquat detoxicative system in the mouse liver postmitochondrial fraction. *Archives of Biochemistry and Biophysics*, *402*(1), 149–157.
42. Han, J.-F. (2006). Effect of Genetic Variation on Human Cytochrome P450 Reductase-Mediated Paraquat Cytotoxicity. *Toxicological Sciences*, *91*(1), 42–48.
43. Elbaz, A., Levecque, C., Clavel, J., Vidal, J. S., Richard, F., Amouyel, P., … Tzourio, C. (2004). CYP2D6 Polymorphism, Pesticide Exposure, and Parkinson's Disease. *Annals of Neurology*, *55*(3), 430–434.
44. Huang, C. L., Chao, C. C., Lee, Y. C., Lu, M. K., Cheng, J. J., Yang, Y. C., … Huang, N. K. (2016). Paraquat Induces Cell Death Through Impairing Mitochondrial Membrane Permeability. *Mol Neurobiol*, *53*(4), 2169–2188.
45. Thomas, B., & Flint Beal, M. (2007). Parkinson's disease. *Human Molecular Genetics*, *16*(R2), 183–194.
46. Djarmati, A., Hedrich, K., Svetel, M., Schäfer, N., Juric, V., Vukosavic, S., … Kostic, V. (2004). Detection of Parkin (PARK2) and DJ1 (PARK7) mutations in early-onset Parkinson disease: Parkin mutation frequency depends on ethnic origin of patients. *Human Mutation*, *23*(5), 525.
47. Bandopadhyay, R., Kingsbury, A. E., Cookson, M. R., Reid, A. R., Evans, I. M., Hope, A. D., … Lees, A. J. (2004). The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain*, *127*(2), 420–430.
48. Canet-Avilés, R. M., Wilson, M. a, Miller, D. W., Ahmad, R., McLendon, C., Bandyopadhyay,

S., … Cookson, M. R. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(24), 9103–9108.

49. Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S. M. M., Takahashi, K., & Ariga, H. (2004). DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Reports*, *5*(2), 213–218.
50. Shendelman, S., Jonason, A., Martinat, C., Leete, T., & Abeliovich, A. (2004). DJ-1 Is a redox-dependent molecular chaperone that inhibits α-synuclein aggregate formation. *PLoS Biology*, *2*(11).
51. Clements, C. M., McNally, R. S., Conti, B. J., Mak, T. W., & Ting, J. P. (2006). DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc Natl Acad Sci U S A*, *103*(41), 15091–15096.
52. Mccoy, M. K., & Cookson, M. R. (2011). DJ-1 regulation of mitochondrial function and autophagy through oxidative stress. *Autophagy*, *5*, 1–2.
53. Maita, C., Maita, H., Iguchi-Ariga, S. M. M., & Ariga, H. (2013). Monomer DJ-1 and Its N-Terminal Sequence Are Necessary for Mitochondrial Localization of DJ-1 Mutants. *PLoS ONE*, *8*(1).
54. Ottolini, D., Calì, T., Negro, A., & Brini, M. (2013). The Parkinson disease-related protein DJ-1 counteracts mitochondrial impairment induced by the tumour suppressor protein p53 by enhancing endoplasmic reticulum-mitochondria tethering. *Human Molecular Genetics*, *22*(11), 2152–2168.
55. Chen, L., Cagniard, B., Mathews, T., Jones, S., Koh, H. C., Ding, Y., … Zhuang, X. (2005). Age-dependent motor deficits and dopaminergic dysfunction in DJ-1 null mice. *Journal of Biological Chemistry*, *280*(22), 21418–21426.
56. Jankovic, J., & Stacy, M. (2007). Medical management of levodopa-associated motor complications in patients with Parkinson's disease. *CNS Drugs*, *21*(8), 677–692.
57. Shoulson, I., Oakes, D., Fahn, S., Lang., William Langston, J., LeWitt, P., … Rudolph, A. (2002).

Impact of sustained deprenyl (selegiline) in levodopa-treated Parkinson' disease: A randomized placebo-controlled extension of Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism trial. *Annals of Neurology, 51*(5), 604-612.

58. Shults, C. W., & Haas, R. (2005). Clinical trials of coenzyme Q10 in neurological disorders. *BioFactors (Oxford, England), 25*(1-4), 117–126.
59. Chase, T, N., Oh, J, D. (2000). Striatal mechanisms and pathogenesis of parkinsonian signs and motor complications. *Annals of Neurology*, 47(4), 122-129.
60. Nutt, J. G., Burchiel, K. J., Comella, C. L., Jankovic, J., Lang, A. E., Laws Jr, E. R., … Schultz, B. (2003). Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology, 60*, 69–73.
61. Gill, S. S., Patel, N. K., Hotton, G. R., O'Sullivan, K., McCarter, R., Bunnage, M., … Heywood, P. (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nature Medicine*, *9*(5), 589–595.
62. Beudel, M., & Brown, P. (2016). Parkinsonism and Related Disorders Adaptive deep brain stimulation in Parkinson ' s disease. *Parkinsonism and Related Disorders, 22*, S123–S126.
63. Shen, Y., Huang, J., Liu, L., Xu, X., Han, C., Zhang, G., … Wang, T. (2016). A Compendium of Preparation and Application of Stem Cells in Parkinson's Disease: Current Status and Future Prospects. *Frontiers in Aging Neuroscience*, *8*(5).
64. Holtzman, D. M., Morris, J. C., & Goate, A. M. (2011). Alzheimer ' s Disease : The Challenge of the Second Century. *Science Translational Medicine*, *3*(77), 1–35.
65. Rhein, V., & Eckert, A. (2007). Effects of Alzheimer's amyloid-beta and tau protein on mitochondrial function -- role of glucose metabolism and insulin signalling. *Archives of Physiology and Biochemistry*, *113*(3), 131–141.
66. Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. (2010). *An overview of APP processing enzymes and products.NeuroMolecular Medicine, 12*(1), 1-12.
67. Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological

alterations in Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, *1*(1), 1–23.

68. Imbimbo, B. P., Solfrizzi, V., & Panza, F. (2010). Are NSAIDs useful to treat Alzheimer's disease or mild cognitive impairment? *Frontiers in Aging Neuroscience*, *2*(5), 1–14.
69. Cleveland, D. W., & Hoffman, P. N. (1991). Neuronal and glial cytoskeletons. *Current Opinion in Neurobiology*, *1*(3), 346–353.
70. Rankin, C. a, Sun, Q., & Gamblin, T. C. (2007). Tau phosphorylation by GSK-3beta promotes tangle-like filament morphology. *Molecular Neurodegeneration*, *2*, 12.
71. Medina, M., Hernández, F., & Avila, J. (2016). New Features about Tau Function and Dysfunction. *Biomolecules*, *6*(2), 21.
72. Pittman, A. M., Fung, H. C., & de Silva, R. (2006). Untangling the tau gene association with neurodegenerative disorders. *Human Molecular Genetics*, *15*(SUPPL. 2), 188–195.
73. Wang, Y., Mandelkow, E. (2016). Tau in physiology and pathology. *Nat Rev Neurosci*, *17*, 22-35.
74. Parsons, C. G., Danysz, W., Dekundy, A., & Pulte, I. (2013). Memantine and cholinesterase inhibitors: Complementary mechanisms in the treatment of Alzheimer's disease. *Neurotoxicity Research*, *24*(3), 358–369.
75. Bayer, T. A., & Wirths, O. (2008). Review on the APP/PS1KI mouse model: Intraneuronal A?? accumulation triggers axonopathy, neuron loss and working memory impairment. *Genes, Brain and Behavior*, *7*(SUPPL. 1), 6–11.
76. Hardy, J. A., & Higgins, G. A. (1992). Alzheimer ' s Disease : The Amyloid Cascade Hypothesis Published by : American Association for the Advancement of Science Alzheimer ' s Disease : The Amyloid Cascade Hypothesis. *Science*, *256*(5054), 184–185.
77. Morris, G. P., Clark, I. a, & Vissel, B. (2014). Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. *Acta Neuropathologica Communications*, *2*, 135.
78. Sheng, M., Sabatini, B. L., & Südhof, T. C. (2012). Synapses and Alzheimer's disease. *Cold Spring Harbor Perspectives in Biology*, *4*(5), 10.
79. Xie, H., Hou, S., Jiang, J., Sekutowicz, M., Kelly, J., & Bacskai, B. J. (2013). Rapid cell death is

preceded by amyloid plaque-mediated oxidative stress. *Proc.Natl.Acad.Sci.U.S.A*, *110*(1091-6490 (Electronic), 7904–7909.

80. Galasko, D., & Montine, T. j. (2010). Biomarkers of Oxidative Damage and Inflammation in Alzheimer´s Disease. *Biomark Med*, *4*(1), 27–36.
81. Geula, C., Wu, C., Saroff, D., Lorenzo, A., Yuan, M., Yankner, B. A. (1998). Age renders the brain vulnerable to amyloid β-protein neurotoxicity. *Nature*, *4*(7), 623-26.
82. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., & Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimerès disease and Down Syndrome. *Proceedings of the National Academy of Sciences of the United States of America. 82*(12): 5245-9.
83. Goate, A., Chartier-Harlin, M. C., … Hardy, J. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimerès disease. *Nature,* 349:704-706.
84. Murrell, J., Farlow, M., … Benson, M. D. (2016). A Mutation in the Amyloid Precursor Protein Associated with Hereditary Alzheimer ' s Disease Author (s): Jill Murrell , Martin Farlow , Bernardino Ghetti and Merrill D . Benson Published by : American Association for the Advancement of Science Stable UR, *254*(5028), 97–99.
85. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva E. A., Levesque, G., Ikeda, M., … St George-Hyslop, P. H. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375(6534): 754-760.
86. Corder, A. E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, W., … Haines, J. L. (2008). Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer ' s Disease in Late Onset Families Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2882127, *261*(5123), 921–923.
87. Levy-lahad, A. E., Wasco, W., Poorkaj, P., Romano, D. M., Pettingell, W. H., Yu, C., … Tanzi, R. E. (2016). Candidate Gene for the Chromosome 1 Familial Alzheimer ' s Disease Locus Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2887712 Accessed : 17-07-2016 23 : 19 UTC Your use of the JSTOR

archive in, 1–6.

88. Kim, H. V. H. Y., Kim, H. V. H. Y., Jo, S., Lee, C. J., Choi, S. Y., Kim, D. J., & Kim, Y. (2015). EPPS rescues hippocampus-dependent cognitive deficits in APP/PS1 mice by disaggregation of amyloid-β oligomers and plaques. *Nature Communications*, *6*, 8997.
89. Pooler, A. M., Polydoro, M., Maury, E. A., Nicholls, S. B., Reddy, S. M., Wegmann, S., … Hyman, B. T. (2015). Amyloid accelerates tau propagation and toxicity in a model of early Alzheimer's disease. *Acta Neuropathologica Communications*, *3*, 14.
90. Salminen, A., Kaarniranta, K., Kauppinen, A., Ojala, J., Haapasalo, A., Soininen, H., & Hiltunen, M. (2013). Impaired autophagy and APP processing in Alzheimer's disease: The potential role of Beclin 1 interactome. *Progress in Neurobiology*, *106-107*, 33–54.
91. Banwait, S., Galvan, V., Zhang, J., Gorostiza, O. F., Ataie, M., Huang, W., … Bredesen, D. E. (2008). C-terminal cleavage of the amyloid-beta protein precursor at Asp664: a switch associated with Alzheimer's disease. *Journal of Alzheimer's Disease : JAD*, *13*(1), 1–16.
92. Galvan, V., Gorostiza, O. F., Banwait, S., Ataie, M., Logvinova, A. V., Sitaraman, S., … Bredesen, D. E. (2006). Reversal of Alzheimer's-like pathology and behavior in human APP transgenic mice by mutation of Asp664. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(18), 7130-7135.
93. Saganich, M. J., Schroeder, B. E., Galvan, V., Bredesen, D. E., Koo, E. H., & Heinemann, S. F. (2006). Deficits in synaptic transmission and learning in amyloid precursor protein (APP) transgenic mice require C-terminal cleavage of APP. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(52), 13428–13436.
94. Nguyen, T. V, Galvan, V., Huang, W., Banwait, S., Tang, H., Zhang, J., & Bredesen, D. E. (2008). Signal Transduction in Alzheimer disease. *J Neurochem*, *104*(4), 1065–1080.
95. Lu, D. C. (2003). Amyloid beta protein toxicity mediated by the formation of amyloid-beta protein precursor complexes. *Ann Neurol*, *54*, 781–789.
96. Shaked, G. M, Kummer, M. P., Lu, D. C., Galvan, V., Bredesen, D. E., Koo, E. H. (2006). Aβ

induces cell death by direct interaction with its cognate extracellular domain on APP (APP 597-624). *FASEB J*, 20(8): 1254-1256.

97. Bredesen, D. E., Mehlen, P., & Rabizadeh, S. (2005). Receptors that mediate cellular dependence. *Cell Death and Differentiation*, *12*(8), 1031–1043.

98. Lourenço, F. C., Galvan, V., Fombonne, J., Corset, V., Llambi, F., & Müller, U. (2009). NIH Public Access. *October*, *16*(5), 655–663.

99. Shankar, G., & Walsh, D. (2009). Alzheimer's disease: synaptic dysfunction and Aβ. *Molecular Neurodegeneration*, *4*(1), 1–13.

100. Zündorf, G., & Reiser, G. (2011). Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection. *Antioxidants & Redox Signaling*, *14*(7), 1275–1288.

101. Morris, J. C., Storandt, M., McKeel, D. W., Rubin, E. H., Price, J. L., Grant, E. A., & Berg, L. (1996). Cerebral Amyloid Deposition and Diffuse Plaques In Normal Aging - Evidence For Presymptomatic and Very Mild Alzheimers Disease. *Neurology*, *46*(3), 707–719.

102. Wang, Q., Li, W., Liu, X. S., Carroll, J. S., Jänne, O. A., Krasnickas, E., … Brown, M. (2014). Amyloid imaging with Carbon 11-labeled Pittsburg compound B for traumatic brain injury. *JAMA Neurol*, *27*(3), 380–392.

103. Washington, P. M., Morffy, N., Parsadanian, M., Zapple, D. N., & Burns, M. P. (2014). Experimental traumatic brain injury induces rapid aggregation and oligomerization of amyloid-beta in an Alzheimer's disease mouse model. *Journal of Neurotrauma*, *31*(1), 125–34.

104. Castellani, R. J., Lee, H.-G., Perry, G., & Smith, M. A. (2006). Antioxidant protection and neurodegenerative disease: the role of amyloid-beta and tau. *American Journal of*

Alzheimer's Disease and Other Dementias, *21*(2), 126–30.

105. Kontush, A. (2001). Amyloid: An antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. *Free Radical Biology and Medicine, 3*(9), 1120-1131.

106. Cai, Q., & Tammineni, P. (2016). Alterations in Mitochondrial Quality Control in Alzheimer's Disease. *Frontiers in Cellular Neuroscience*, *10*(2), 24.

107. Doody, R. S., Raman, R., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., … Mohs, R. (2013). A phase 3 trial of semagacestat for treatment of Alzheimer's disease. *The New England Journal of Medicine*, *369*(4), 341–50.

108. Vladar, E. K., Lee, Y. L., Stearns, T., & Axelrod, J. D. (2015). *Curr Alzheimer Res*, *11*(5), 37-54.

109. Lipton SA (2004). Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx : The Journal of the American Society for Experimental NeuroTherapeutics* 1(1), 101–10.

110. Hughes RE, Nikolick, Ramsay RR (2016) One for All? Hitting Multiple Alzheimer's Disease Targets with One Drug. *Frontiers in Neuroscience* 10, 1-10.

111. Youdim, M. B. H. (2013). Multi target neuroprotective and neurorestorative anti-Parkinson and anti-Alzheimer drugs ladostigil and m30 derived from rasagiline. *Experimental Neurobiology, 22*(1), 1-10.

112. Corbett, A., Pickett, J., Burns, A., Corcoran, J., Dunnett, S. B., Edison, P., … Ballard, C. (2012). Drug repositioning for Alzheimer's disease. *Nature Reviews Drug Discovery*, *11*(11), 833–846.

113. Holscher, C. (2014). First clinical data of the neuroprotective effects of nasal insulin

application in patients with Alzheimer's disease. *Alzheimer's and Dementia*, *10*(1 SUPPL.), 33–37.

114. McCarthy, S., Somayajulu, M., Sikorska, M., Borowy-Borowski, H., & Pandey, S. (2004). Paraquat induces oxidative stress and neuronal cell death; Neuroprotection by water-soluble Coenzyme Q10. *Toxicology and Applied Pharmacology*, *201*(1), 21–31.

115. Jin, H., Kanthasamy, A., Ghosh, A., Anantharam, V., Kalyanaraman, B., & Kanthasamy, A. G. (2014). Mitochondria-targeted antioxidants for treatment of Parkinson's disease: preclinical and clinical outcomes. *Biochimica et Biophysica Acta*, *1842*(8), 1282–94.

116. Spindler, M., Flint Beal, M., & Henchcliffe, C. (2009). Coenzyme Q10 effects in neurodegenerative disease. *Neuropsychiatric Disease and Treatment*, *5*(1), 597–610.

117. Borowy-Borowski, H. ., Sodja, C. ., Docherty, J. ., Walker, P. R. ., & Sikorska, M. . (2004). Unique technology for solubilization and delivery of highly lipophilic bioactive molecules. *Journal of Drug Targeting*, *12*(7), 415–424.

118. Sikorska, M., Borowy-Borowski, H., Zurakowski, B., & Walker, P. R. (2003). Derivatised alpha-tocopherol as a CoQ10 carrier in a novel water-soluble formulation. *BioFactors (Oxford, England)*, *18*(1-4), 173–83.

119. Naderi, J., Somayajulu-Nitu, M., Mukerji, A., Sharda, P., Sikorska, M., Borowy-Borowski, H., … Pandey, S. (2006). Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis*, *11*(8), 1359–1369.

120. Sandhu, J. K., Pandey, S., Ribecco-Lutkiewicz, M., Monette, R., Borowy-Borowski, H., Walker, P. R., & Sikorska, M. (2003). Molecular mechanisms of glutamate neurotoxicity in mixed cultures of NT2-derived neurons and astrocytes: Protective effects of coenzyme Q10.

Journal of Neuroscience Research, *72*(6), 691–703.

121. Ma, D., Stokes, K., Mahngar, K., Domazet-Damjanov, D., Sikorska, M., & Pandey, S. (2014). Inhibition of stress induced premature senescence in presenilin-1 mutated cells with water soluble Coenzyme Q10. *Mitochondrion*, *17*, 106–115.

122. Somayajulu-Niţu, M., Sandhu, J. K., Cohen, J., Sikorska, M., Sridhar, T. S., Matei, A., … Pandey, S. (2009). Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of coenzyme Q10. *BMC Neuroscience*, *10*, 88.

Web links:

http://www.pdf.org/en/parkinson_statistics

http://www.alz.org/dementia/down-syndrome-alzheimers-symptoms.asp

Chapter 2

Orally delivered water soluble Coenzyme Q_{10} (Ubisol-Q_{10}) blocks on-going neurodegeneration in rats exposed to paraquat: potential for therapeutic application in Parkinson's disease

2.1 Synopsis:

Paraquat, still used as an herbicide in some parts of the world, is now regarded as a dangerous environmental neurotoxin and is linked to the development Parkinson's disease (PD). Paraquat interacts with cellular redox systems and causes mitochondrial dysfunction and the formation of reactive oxygen species, which in turn, plays a crucial role in the pathophysiology of PD. Various antioxidant therapies have been explored with the expectations that they deliver health benefits to the PD patients, however, no such therapies were effective. Here we have tested the neuroprotective efficacy of a novel water-soluble CoQ_{10} (Ubisol-Q_{10}), in a rat model of paraquat-induced neurodegeneration in order to evaluate its potential application in the management of PD. We have developed a rat model of progressive nigrostriatal degeneration by giving rats five intraperitoneal injections of paraquat (10 mg/kg/injection), once every five days. Neuronal death occurred over a period of 8 weeks with close to 50% reduction in the number of tyrosine hydroxylase-positive cells. Ubisol-Q_{10}, at 6 mg CoQ_{10}/kg body weight/day, was delivered as a supplement in drinking water. The intervention began after the completion of paraquat injections when the neurodegenerative process had already began and about 20% of TH-positive neurons were lost. Ubisol-Q_{10} treatment halted the progression of neurodegeneration and remaining neurons were protected. The outcomes were evaluated based on the number of surviving tyrosine hydroxylase-positive neurons in the substantia nigra region and improved motor skills in response to the Ubisol-Q_{10} intervention. To maintain this neuroprotection, however, continuous

Ubisol- Q_{10} supplementation was required, if withdrawn, the neuronal death pathway resumed, suggesting that the presence of CoQ_{10} was essential for blocking the pathway. The CoQ_{10}, given orally as Ubisol-Q_{10} in drinking solution, was effective in blocking the progression of neurodegeneration when administered therapeutically (post-toxin injection), at a much lower concentration than other previously tested oil soluble formulations and well within the acceptable daily intake of 12 mg/kg/day. Such unprecedented neuroprotection has never been reported before. These results are very encouraging and suggest that Ubisol-Q_{10} should be further tested and developed as a therapy for halting the progression of PD.

2.2 Introduction:

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterised by the loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc) region of the brain. PD affects approximately 1–2% of the population, above the age of 55 and with the steady growth of the ageing population, disease management is a growing concern for neurologists and other physicians. By the time the characteristic features of PD such as bradykinesia, rigidity, postural instability, and resting tremor become obvious, approximately 60-70% of DA neurons in the SNpc are lost [1]. Currently, there is no therapy available to halt the progression of this neurodegeneration. It has been possible, however, to alleviate the symptoms of the disease by providing dopamine replacement. Administration of levodopa is the most commonly utilized treatment for symptomatic relief [2], yet its prolonged application leads to drug induced dyskinesia, which severely affects the patient's quality of life.

In the majority of cases the cause of PD remains unknown, but factors contributing to the pathogenesis of the disease are extensively studied. PD can be caused by environmental factors such as exposure to herbicides and pesticides or by genetic factors linked to gene mutations that increase the susceptibility to PD [3]. Although these genetic defects account for only 10% of PD cases, their identification brings about a better understanding of the disease pathophysiology and its progressive nature [4]. It is known that classical symptoms of PD can be caused by exposure to neurotoxin MPTP. In 1983 Langston's group found PD like symptoms in young drug addicts who consumed heroin containing MPTP, a by-product in the synthesis of a synthetic heroin [5]. Later it was shown that MPTP injections cause selective loss of DA neurons in the SNpc region

of certain strains of mice thereby creating animal models of PD [6-9]. Although MPTP is not an environmental toxin and humans are not commonly exposed to it, several epidemiological studies reveal a link between the use of herbicides and pesticides such as paraquat (PQ), maneb and rotenone and the incidence of PD [10]. It was subsequently discovered that the active metabolite of MPTP, MPP + and PQ have structural similarity. They enter the DA neurons via the dopamine transporter as well as trigger neurodegeneration [11]. Three independent studies in Texas, Taiwan and California show that exposure to PQ indeed causes an increased susceptibility to PD [12,13]. In rodents, PQ exposure leads to the loss of DA neurons in the SNpc region of the brain in a time and dose dependent manner [14]. Therefore, rat and mouse models of PQ-induced neurodegeneration have been developed to study the pathophysiology of the disease and to develop successful treatment strategies.

One consistent finding between the PD patients and animal models of PD (MPTP, PQ, rotenone) is the malfunctioning of complex I of the electron transport chain suggesting clearly, that mitochondrial dysfunction is at the centre of PD pathophysiology [4]. It seems that a blockade of complex I of the oxidative phosphorylation pathway by these toxins and the inability of DA neurons to cope with the excess of generated free radicals are the triggers of neuronal death. Therefore, it should be possible to interfere with the progression of neurodegenerative processes by applying antioxidants, such as CoQ_{10} and/or Vitamin E, which are capable of reducing the levels of free radicals. However, both these antioxidants are lipid soluble compounds, characterized by limited bioavailability and difficult to deliver systemically, especially to the brain. Numerous studies have shown that CoQ_{10} is effective in preventing cell death caused by toxins such as PQ, however, very high doses of CoQ_{10} (from oil soluble formulation available on the market) are required to provide neuroprotection *in vivo* [15]. Our collaborators at NRC

(Ottawa, ON) have developed a nanomiscelle formulation of CoQ_{10} (Ubisol-Q_{10}), which appears water soluble and contains CoQ_{10} and a derivatized form of α-tocopherol (vitamin E) [16,17]. The solubilization of CoQ_{10} is achieved due to amphipathic properties of PEG-derivatised α-tocopherol allowing the formation of stable and water soluble nanomicelles [16,17]. This formulation has been tested in several cell culture models and it has been shown to be efficient in protecting neurons from the toxic effects of PQ [18]. It has also been tested *in vivo* in rats exposed to PQ [14]. Prophylactic application of Ubisol-Q_{10} in this rat model of PD, provided as drinking solution prior to the PQ exposure and throughout the duration of experiments, clearly confirm its neuroprotective efficacy against PQ. The beneficial effects were achieved at a much lower dose of CoQ_{10} (6 mg/kg b.w.) compared to oil soluble formulation, which was used at 200 – 1600 mg/kg/day in mice [19].

Since PD is diagnosed when the symptoms appear and the neurodegeneration is already in progress, the prophylactic treatments, especially in sporadic cases of PD, are not relevent. Therefore, we designed a study to examine whether a therapeutic intervention with Ubisol-Q_{10} in rats already exposed to PQ could halt the on-going neurodegeneration and behavioural deterioration. Furthermore, we investigated whether a sustained supplementation of Ubisol-Q_{10} was needed to maintain the neuroprotection. Here we present our data showing that oral delivery of Ubisol-Q_{10}, starting after the PQ injections, did halt neurodegeneration and prevented a loss of normal motor skills.

2.3 Materials and methods:

Animal care

All procedures involving animals were carried out in accordance with the Canadian Council for Animal Care guidelines and approved by the University of Windsor's Animal Care Committee. Three months old male Long Evans Hooded rats were purchased from Charles River Laboratories. Rats in the same treatment group were housed together (3–4 per cage) for convenience and in order to prevent any hierarchy that could arise due to the extent of neurodegeneration. The rooms that housed the rats were maintained at 20℃ in a reversed 12 h:12 h dark light cycle.

Paraquat neurotoxicity model and Ubisol-Q_{10} treatments

Rats received 5 intraperitoneal injections of PQ at a dose of 10 mg/kg body weight/injection dissolved in phosphate buffered saline (PBS), one injection every 5 days over a period of 20 days. Control rats received intraperitoneal injections of PBS alone. Brain tissue was examined immediately after the last PQ injection and, subsequently, 4 weeks and 8 weeks later. Supplementation of drinking water with Ubisol-Q_{10} at a concentration of 200 μg/ml (equivalent to 50 μg CoQ_{10}/ml) begun on the day of the last PQ injection and it was continued for either 4 weeks or 8 weeks. Fresh drinking solutions were provided every second day. Sterile stock solution of Ubisol-Q_{10} at 200 mg/ml (equivalent to 50 mg CoQ_{10}/ml) was provided by Zymes LLC (Hasbrouck, NJ). At the conclusion of experimental treatments, rats were perfused with Tyrodes buffer containing heparin, the tissues were fixed with 10% formalin, and the brains extracted and stored in the 10% formalin until processing for immunohistochemistry.

CoQ_{10} bioavailability study

Rats were deprived of water for a period of 24 hours prior to a full 1 h access to drinking water supplemented with Ubisol-Q_{10} at a concentration equivalent to 50 μg CoQ_{10}/ml. The rats were sacrificed at 1, 3 and 6 h after the feeding. Brain tissue was collected and CoQ_{10} content was measured as previously described [27,28,29]. Briefly, samples were homogenized in cold PBS and subjected to repeated freezing/thawing steps to disrupt protein/lipid complexes. CoQ_{10} was extracted and analysed by HPLC following separation on a TSK-GEL ODS-100S column (4.6 mm × 150 mm, 7 μ particle size, TOSOH Biosep LLC, Montgomeryville), equipped with a 1 mm C18 guard column (Optimize Technologies Inc., Oregon City, OR). Absorbance at 275 nm was monitored and recorded using Beckman System Gold Software.

Toxicity study

A group of rats (4 rats) were kept for 2.5 months on drinking water supplemented Ubisol- Q_{10} at a concentration 2 μg/ml (equivalent to 50 μg/ml of CoQ_{10}) or 10 times the dose used in the neuroprotection study. Animals were weighed once a week to ensure their health. The rats were then perfused with heparin containing Tyrodes buffer and formalin fixed tissue – heart, lung, liver and kidney were sent to the Animal Health Laboratory, University of Guelph. Hematoxylin & Eosin -stained histological sections of the tissues were evaluated by a board-certified veterinary pathologist.

Rat horizontal beam walking test

All rats were assessed for performance on a horizontal beam-walking test for motor skills/motor deficits as measured by leg slips. The aluminium beam was 1.68 metres in length, 2 centimetres

in width and 0.75 metres from the ground. A mirror was placed behind the beam, measuring 1.78 metres in length and 0.3 metres in height. Four weeks after the last injection, rats underwent one trial per day for four consecutive days (one training trial and three test trials). Eight weeks after the last injection another three test trials were performed (one trial per day). In the training trial, rats ran down the beam to the holding cage on a flat platform three times, each time with different distances between the holding cage and starting position. The first position was a quarter of the beam length, the second was half, and the last was the entire distance of the beam. This last distance is where mice were placed for the subsequent test trails.

Rats received a small slice of apple in the holding cage located on a table at the end of the beam. The rat had up to 2 minutes to cross the beam. The test trials were recorded using a standard video camera, located 2 metres perpendicular to the beam. The number of hind leg slips made from either leg during each test trial was later noted from viewing the recorded video clips. The number of limb slips for each rat was summed over the three test sessions in each phase because rats made too few slips in each session to analyse this behaviour over trials within each session. The statistical analysis of each rat's total number of leg slips over each test series was carried by a two-way. Effects from these analyses were considered significant at $p < 0.05$. Post-hoc comparisons between groups at each test phase were carried out by Least Significant Difference multiple comparisons and significant differences between groups were considered at $p < 0.05$ (one-tail) based on the prediction that rats not given post-injection Ubisol-Q_{10} in their drinking water would show deficits associated with PQ-induced neurodegeneration.

Immunohistochemistry

The brains kept in 10% formalin were transferred to 30% sucrose (w/v) three days before sectioning. The midbrain region was sectioned at a thickness of 30 μm and 64 sections were collected in total. The sections were subjected to immunohistochemistry with anti-tyrosine hydroxylase antibody (1:1000 dilution) purchased from Pel-Freeze Biologicals, USA. Prior to overnight incubation with the primary antibody at 4 ˚C, the sections were incubated in 1% H_2O_2 for 5 minutes to block endogenous peroxidase, DAKO universal blocking solution (purchased from Diagnostics Canada Inc., Mississauga) for 30 minutes and in normal goat serum (prepared as per instructions on anti-rabbit Vecstatin ABC Kit, Vector Laboratories) for 30 minutes in order to block the binding of non-specific goat IgG. The sections were washed in Tris buffered saline (TBS) twice for 5 minutes between the blocking steps in order to remove any excess blocking reagents. After overnight incubation the slides were washed in TBS twice and incubated in biotinylated anti-rabbit IgG raised in goat (anti-rabbit Vecstatin ABC Kit) for 1.5 hours. The slides were again washed twice for 5 minutes with TBS, following which they were incubated in avidin biotin complex (ABC reagent) for 45 minutes. Then the two five minute washes with TBS were repeated, and the peroxidase substrate 3, 3′ diaminobenzidine (DAB) prepared as per the instructions to specifically stain the DA neurons. The sections were then dehydrated with 95% ethanol and xylene and coverslipped to be able to visualise under the microscope.

The number of TH-positive neurons in the SN were counted using the Stereology Software provided by the Stereology Resource Center, Chester, MD as previously described [30,31] . The

SN region was outlined at low magnification and the neurons were counted at high magnification.

Statistical analysis

Differences among means were analysed using one way analysis of variance (ANOVA) and pairwise comparisons between means were analyzed by *post hoc* Bonferroni's multiple comparison test. The software used was GraphPad Prism ver. 4.0.

2.4 Results:

Brain delivery of Coenzyme Q10

The brain CoQ_{10} levels were measured in rats which were given a 1 h access to Ubisol-Q_{10} supplemented water (at a concentration of 50 μg /ml) after a 24 h period of water deprivation. During this time rats drank on average 10 ml of solution containing 500 μg of CoQ_{10}. Animals were sacrificed at different time points after the Ubisol-Q_{10} intake, CoQ_{10} was extracted and analysed by HPLC. The results are shown in Figure 3. We observed a modest, but time dependent elevation of CoQ_{10} in the brain, peaking at 3 h post-feeding, with values 30-50% higher than the basal level, suggesting that its transfer to the brain parenchyma and subsequent metabolic turnover was taking place. A question whether these changes were sufficient to achieve a therapeutic neuroprotection against PQ was examined below (Figures 4, 5 and 6).

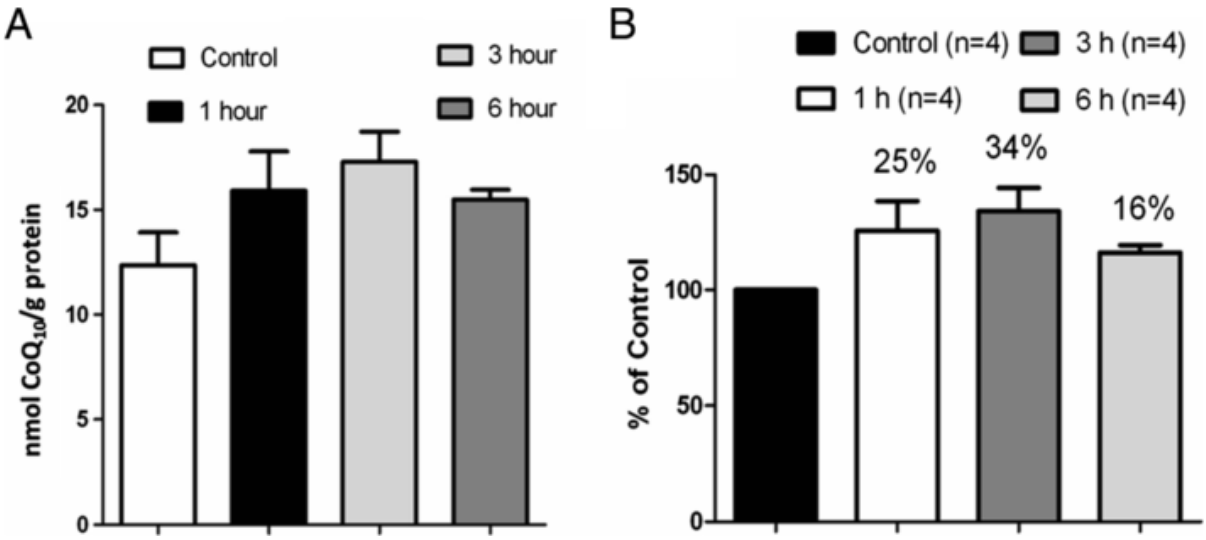


Figure 3 Bioavailability of Ubisol – Q_{10}. (A) There is a gradual increase in the levels of CoQ_{10} in the rat brains that were sacrificed at the 1 and 3 hour time points following the 1 hour feeding with Ubisol – Q_{10} supplemented drinking water after the 24 hour water starvation in comparison with the control group that was fed with regular drinking water for 1 hour instead. The control group was sacrificed 1 hour following the water feeding. (B) There is a 25% increase in the level of CoQ_{10} at the 1 hour time point, 34% at the 3 hour time point and a decline to 16% at the 6 hour time point.

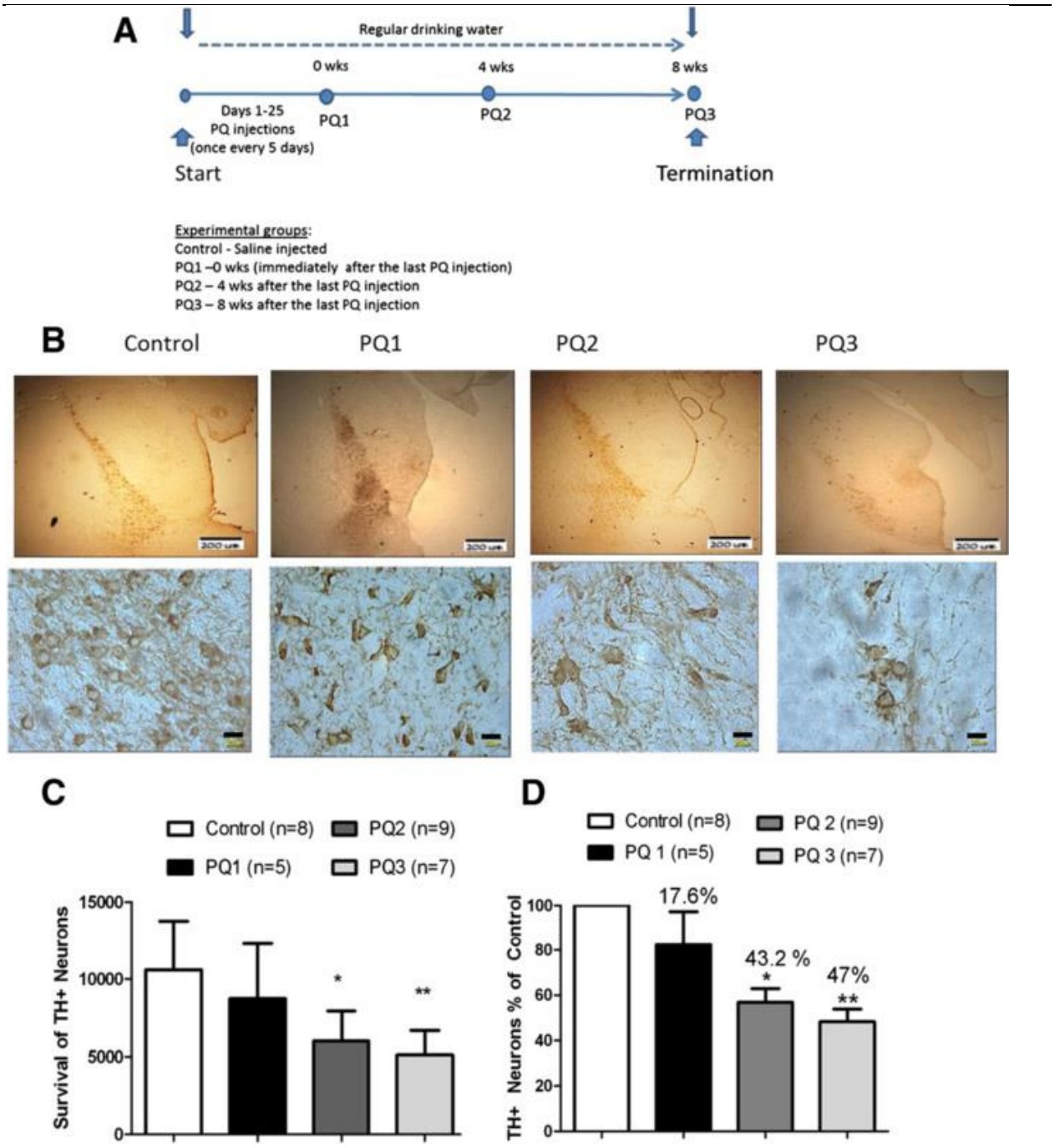
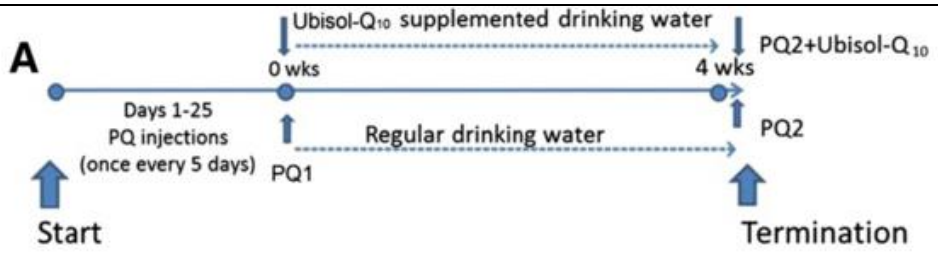
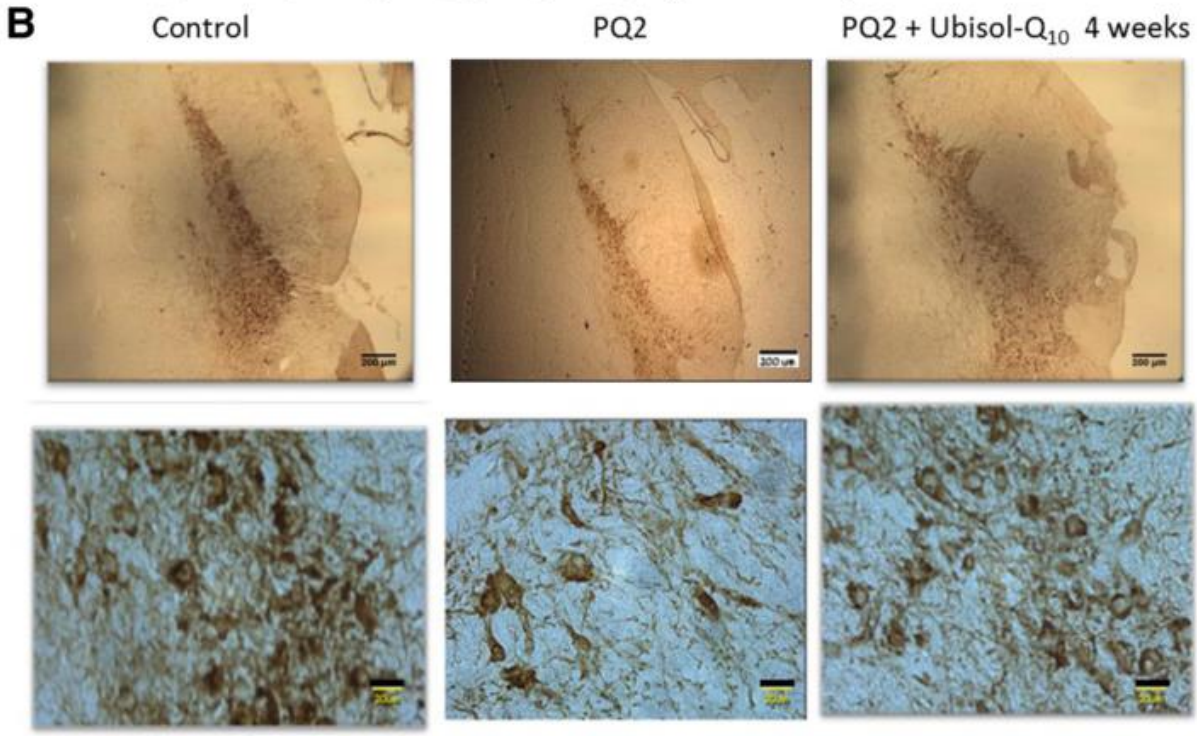


Figure 4 Progressive loss of DA neurons following PQ injections. (A) The experimental plan demonstrating the injection regime and treatment schedule. Immunohistochemistry performed using anti-tyrosine hydroxylase antibodies on the brain sections of animals. (B) Representative images of midbrain sections showing TH positive neurons at lower and higher magnifications from saline injected control group rats, PQ injected rats dissected 24 hours after the last injection (PQ1 group), PQ injected rats dissected four weeks after the last injection (PQ2 group), PQ injected rats dissected eight weeks after the last injection (PQ3 group). The area of SN which is to be counted is selected in every 6^{th} section of the midbrain (sectioned at 30 microns thickness).

(C) The total number of TH positive neurons in the SNpc region was counted at higher magnification using the stereology software purchased from the Stereology Resource Centre, Inc., Florida. There is a significant decrease in the number of TH - positive neurons (* $p < 0.05$) in the rats sacrificed four weeks and (** $p < 0.05$) eight weeks after the last injection in comparison with saline injected control group and no significance in rats sacrificed immediately after the last injection verses saline injected control groups. (D) The percentage decrease in TH positive neurons between the saline injected control and PQ groups. There is 43.3% and 47% decrease in the TH positive neurons in the rats sacrificed four and eight weeks after the last injection and only a 17.8% decrease in the number of TH positive neurons in the rats sacrificed 24 hours after the last injection. The bars in the upper (low magnification) panel are 200 μm and in the lower (high magnification) panel 20 μm.



Experimental groups:
Control- saline injected/regular drinking water
PQ2 – 4wks post PQ injections/regular drinking water
PQ2+Ubisol-Q_{10}– 4 wks post PQ injections/Ubisol-Q_{10}supplemented drinking water



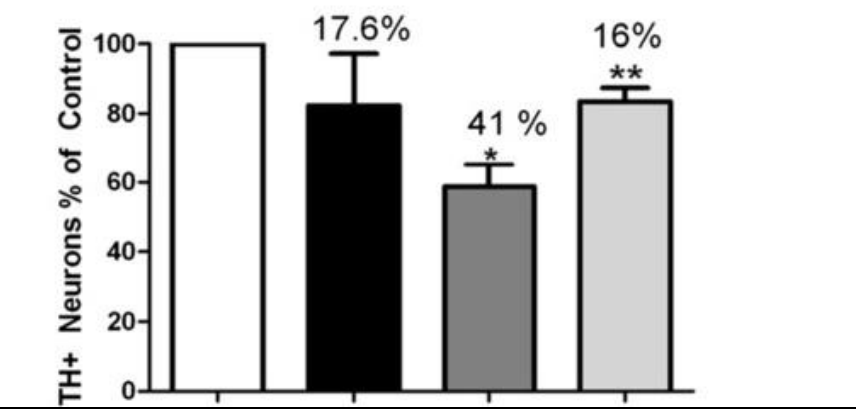


Figure 5 Halting the progression of neurodegeneration by Ubisol – Q_{10}. (A**)** The experimental plan demonstrating the injection regime and treatment schedule. Immunohistochemistry performed using anti-tyrosine hydroxylase antibodies on the brain sections of animals. (B) Representative images of midbrain sections showing TH positive neurons at lower and higher magnifications from saline injected control group rats, PQ injected rats fed with regular drinking water dissected immediately after the last injection (PQ1), PQ injected rats fed with regular drinking dissected 4 weeks after last injection (PQ2), PQ injected rats fed with Ubisol – Q_{10} supplemented drinking water after last injection and dissected 4 weeks after last injection (PQ2 + Ubisol- Q_{10} 4 weeks). The area of SN which is to be counted is selected in every 6^{th} section of the midbrain (sectioned at 30 microns thickness). The total number of TH positive neurons in the SNpc region was counted at high magnification using the stereology software purchased from the Stereology Resource Centre, Inc., Florida. (C) The percentage decrease in TH positive neurons between the saline injected control group and the PQ injected treated and untreated groups. There is a significant 41% decrease in the TH positive neurons in the PQ2 group in comparison with the saline injected control group (*$p < 0.05$), whereas there is loss of 17% neurons in the Ubisol – Q_{10} treated group verses the control (**$p < 0.05$) indicating significant neuroprotection when compared to the PQ2 group. The bars in the upper (low magnification) panel are 200 μm and in the lower (high magnification) panel 20 μm.

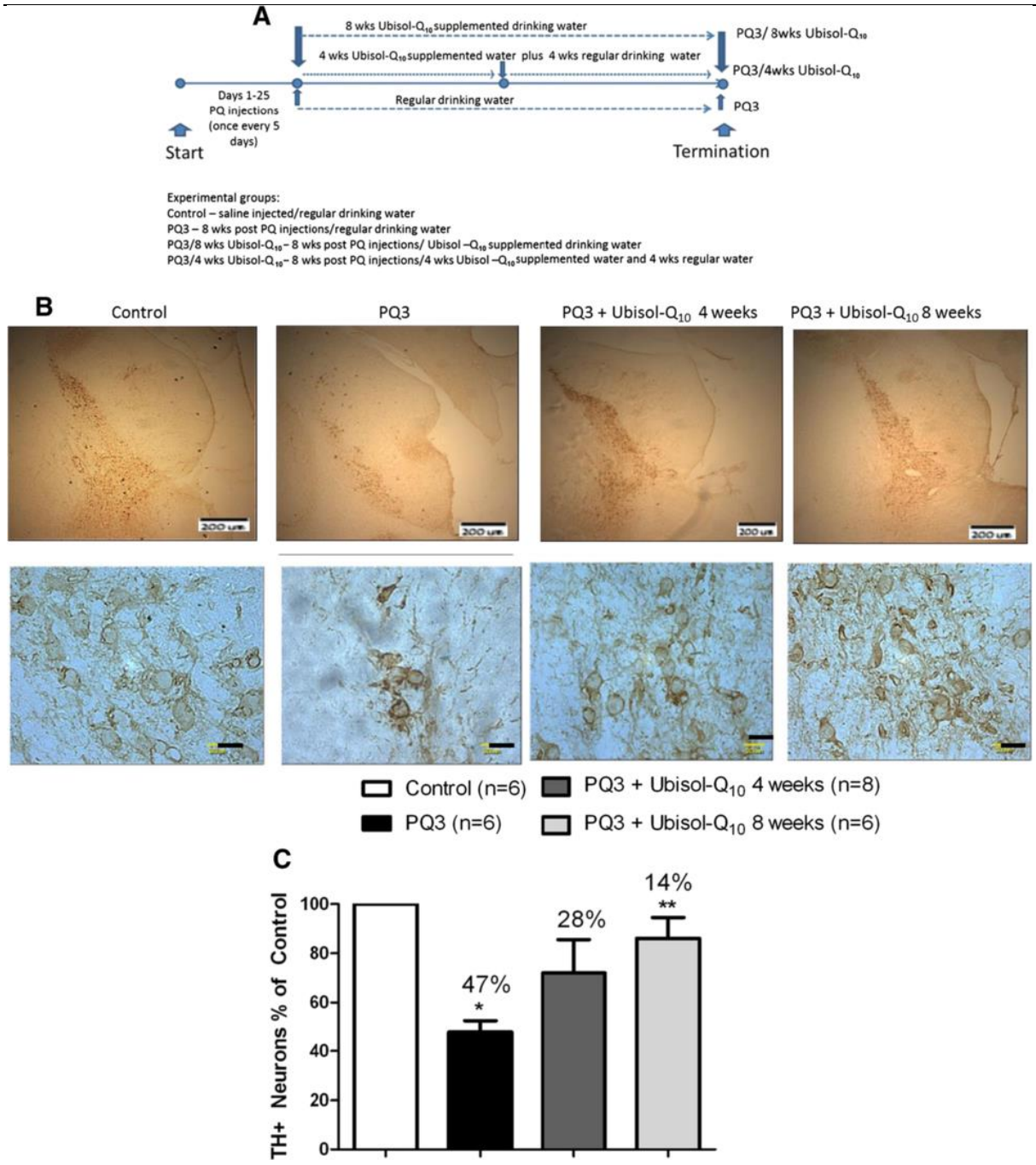


Figure 6 Sustained feeding of Ubisol-Q_{10} is needed for neuroprotection. (A) The experimental plan demonstrating the injection regime and treatment schedule. (B) Immunohistochemistry performed using anti-tyrosine hydroxylase antibodies on the brain sections of animals. Representative images of midbrain sections showing TH positive neurons at lower and higher magnifications from saline injected control group rats, PQ injected rats fed with regular drinking water dissected 8 weeks after last injection (PQ3), PQ injected rats fed with Ubisol – Q_{10} supplemented drinking water for 4 weeks followed by regular drinking water for 4 weeks after last injection with PQ and dissected 8 weeks after last injection (PQ3 + Ubisol – Q_{10} 4 weeks group), PQ injected rats fed with Ubisol – Q_{10} supplemented drinking water for 8 weeks after last

injection with PQ and dissected 8 weeks after last injection (PQ3 + Ubisol – Q_{10} 8 weeks group). The area of SN which is to be counted is selected in every 6^{th} section of the midbrain (sectioned at 30 microns thickness). The total number of TH positive neurons in the SNpc region was counted at high magnification using the stereology software purchased from the Stereology Resource Centre, Inc., Florida. (C) The percentage decrease in TH positive neurons between the saline injected control and PQ injected treated and untreated groups. There is a significant 47% decrease in the TH positive neurons in the PQ3 group in comparison with the saline injected control group (*$p < 0.05$) and loss of 28% neurons in the PQ3 + Ubisol-Q_{10} 4 weeks group. A 14% decrease in TH positive neurons is seen in the treated PQ3 + Ubisol-Q_{10} 8 weeks (**$p < 0.05$) indicating significant neuroprotection when compared to the PQ3 group. The bars in the upper (low magnification) panel is 200 μm and in the lower (high magnification panel) 20 μm.

Paraquat model of progressive neurodegeneration

Long Evans Hooded rats were used to develop a model of progressive neurodegeneration. The rats were given 5 intraperitoneal injections of PQ (10 mg/ kg b.w./injection), one injection every five days over a period of 20 days. Animals were sacrificed at different time points for up to 8 weeks post-PQ exposure. Midbrain sections were prepared, immunostained with anti-tyrosine hydroxylase antibody and TH- positive neurons were counted using a stereologer, in an unbiased manner. As shown in Figure 4, a substantial percentage of DA neurons, i.e., close to 18%, were lost during the PQ injection period (PQ1 group). The neurons continued to die over the next several weeks reducing the number of TH-positive neurons by 43% at the end of week 4 (PQ2 group) and 47% by end of week 8 (PQ3 group) post-PQ exposure. The results confirmed that PQ triggered progressive neurodegeneration in this strain of rats, mimicking to some extent, changes occurring in Parkinsonian patients. This model is probably closest to what happens in humans as one month in a rat's lifetime is equivalent to 2.5 years in human [20]. Therefore, this model was used to assess a therapeutic neuroprotection of CoQ_{10}.

Therapeutic intervention with Ubisol-Q_{10}

We have previously shown that prophylactic treatment with Ubisol – Q_{10} effectively protects rat brain from PQ toxicity [18]. In this study we applied the Ubisol-Q_{10} intervention after the completion of PQ injections. By this time, neurodegenerative processes in the brain had already triggered (Figure 4). The PQ-treated group of rats was placed on Ubisol-Q_{10} supplemented drinking water (containing 50 μg/ml of CoQ_{10}) for 4 weeks (PQ2 + 4 wks Ubisol-Q_{10} group). This treatment began when nearly 18% of SN neurons were already lost (PQ1 group, Figure 4), but the question was whether the remaining vulnerable neurons could be saved. The generated

data is summarized in Figure 3. The midbrain sections were immunostained with anti- TH antibodies, and the stained neurons were counted using a stereologer in an unbiased manner. As shown above (Figure 5), the PQ- treated rats drinking regular water (PQ2 group) lost over 40% of DA neurons over the period of 4 weeks, whereas rats drinking Ubisol – Q_{10} lost less than 20% (PQ2 + 4 wks Ubisol-Q_{10} group). Clearly, this Ubisol-Q_{10} treatment saved close to 17% of neurons which would have otherwise died as the consequence of PQ exposure. This unprecedented neuroprotection has never been reported in animal models of neurotoxicity and could offer hope to PD patients for better disease management.

We then examined how long Ubisol – Q_{10} supplementation would be required to maintain neuroprotection. In this set of experiments the PQ treated rats were either kept on Ubisol – Q_{10} for the full 8 weeks post-PQ or the treatment was withheld after 4 weeks and rats were given regular tap water for the additional 4 weeks (8 weeks total). There was a significant loss of DA neurons, approximately 47% in comparison with the saline injected control group indicating progressive neurodegeneration over a period of eight weeks (Figure 6). There was also significant neuroprotection in the rats that received the Ubisol – Q_{10} supplemented drinking water for eight weeks post injections. Since the Ubisol – Q_{10} intervention began after nearly 15% of DA neurons were already killed (Figure 4) no further loss of neurons was observed as a result of this intervention (Figure 6, only 14% of neuronal loss recorded). However, if the treatment was withheld after 4 weeks, the neurodegeneration resumed as evidenced by the reduced number of surviving neurons in this experimental group comparing to the group receiving Ubisol – Q_{10} for 8 weeks (28% versus 14%, respectively). Therefore, continuous Ubisol – Q_{10} supplementation was required to maintain the achieved level of neuroprotection.

Behaviour results

Deficiency in the motor function is a hallmark of PD. Next, we asked whether loss of DA neurons correlated with the deficiency of behavioural motor function following PQ treatment, and whether motor deterioration was blocked by Ubisol-Q_{10} treatment. We applied a horizontal beam walking test as described in the method section. Results shown in Figure 7 indicated that the PQ3 group made more leg slips than either the control or the PQ3 + Ubisol-Q_{10} 8 weeks in both the test phases or than the PQ3+ Ubisol-Q10 4 weeks group in the first test phase. The PQ3 + Ubisol-Q_{10} 4 weeks increased its leg slips to the elevated levels of the PQ3 group in the second test phase. Multiple comparisons between groups confirmed that the number of leg slips of the PQ3 group was significantly greater than those of the other three groups ($p < 0.05$) in the 1st test phase but only remained significantly greater than that of the PQ3+/Ubisol-Q_{10} 8 weeks group in 2nd test phase ($p = .036$). Thus even though the observed groups by test phase interaction were not significant, multiple comparison between groups reveal that only those rats that received Ubisol –Q_{10} in their drinking water over the complete post injection period maintained their superior performance similar to that of rats that were not exposed to potential neurodegenerative effects of PQ. From a behavioral aspect, treatment with the neuroprotectant agent only half way through the post-injection period was not sufficient to maintain its effect to the end of the experiment.

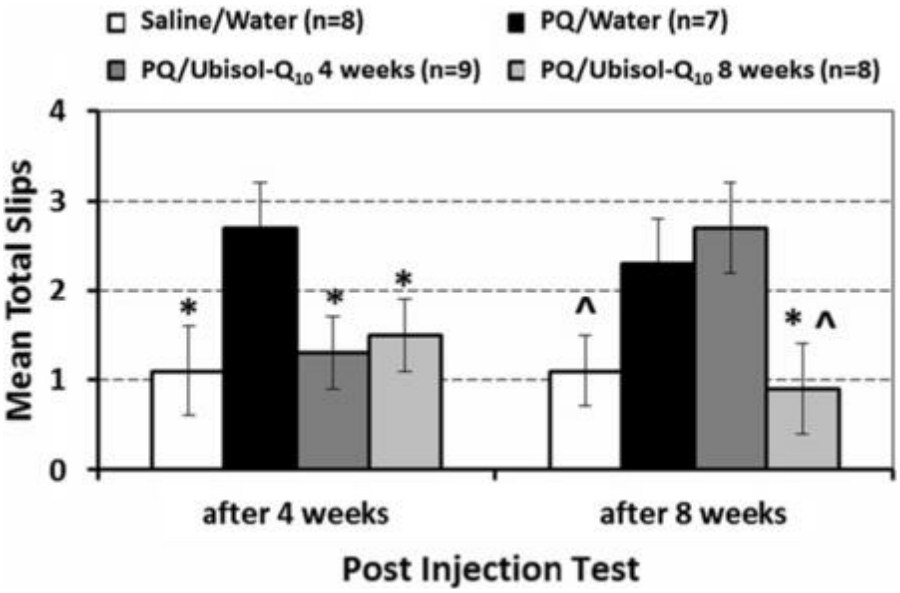


Figure 7 Mean total number of hind leg slips over three sessions in each post-injection test for each Injection/Treatment group. Vertical error lines represent ± SEM. Significant difference ($p < 0.05$) between the control or either of the two PQ/ Ubisol – Q_{10} groups and the PQ3 group in each injection test phase are designated by ***** and between the control and the PQ/Ubisol-Q_{10} 8 weeks group and the PQ3 in the second injection phase by ^.

Toxicity assessment

The data presented above (Figure 6) clearly indicated that long-term treatments with Ubisol – Q_{10} would be required to maintain the neuroprotection. To ensure safety of such long treatments, we carried out a pilot toxicity study, in which rats were maintained on drinking water supplemented with Ubisol – Q_{10} at a dose 10 times higher (60 mg/kg/day) than that used for neuroprotection (6 mg/kg/day) for 2.5 months. During this time, the Ubisol – Q_{10} treated rats never displayed any signs of discomfort, no change in eating, drinking, grooming habits and no difference in body weight in comparison with rats drinking regular tap water over the same time period. At the conclusion of the experiments several tissues were collected and sent for histopathological examination by a board certified pathologist at the University of Guelph. No overt lesions of toxicological significance were observed in the Ubisol-Q_{10}-treated animals (data not shown).

2.5 Discussion:

For the first time ever, we have effectively established an animal model, using PQ, which accurately demonstrates the chronic and progressive neurodegeneration similar to that in PD patients. Compared to an acute model, our model effectively replicates PD by having slow, chronic degeneration of DA neurons in SNpc. However, progressive neurodegeneration has also been observed with continuous infusion of MPTP using an osmotic pump [21]. The progressive loss was evident in our results over the observed time span, with a decreased number of neurons remaining at each subsequent time interval. Our model allows us to intervene with treatment at any point after the initiation of the disease. Due to this we were also able to demonstrate for the first time, that when Ubisol-Q_{10} is administered therapeutically it effectively halts the progression of the neurodegeneration, even at low dosages. After demonstrating the success Ubisol-Q_{10} has in protecting the neurons therapeutically, it was shown that this treatment needs to be continuous. Halting the administration of the treatment would result in a continuation of the neurodegeneration initiated by the PQ toxin during the injection regime. With bioavailability data, we were also able to show that Ubisol-Q_{10} is effective at increasing the CoQ_{10} levels in the brain. All of the results were supported using behavioural, histochemical, and biochemical methodology.

An animal model of PD was developed when the neurotoxin MPTP was found to cause PD like symptoms and loss of DA neurons by blocking complex I of the electron transport chain [7]. MPTP establishes an acute model of PD, which is not realistic to the natural progression of the disease. Following this, it was found that environmental toxins, rotenone and PQ, can also block complex I of the electron transport chain. Further supported by epidemiological studies, a link between the use of these pesticides and the incidence of PD was established [22]. The

development of the PQ animal model of PD is more relevant than the acute MPTP model as it more effectively mimics PD in patients.

In previous research, a variety of dosages and injection regimes of PQ have been used in rat models of PD [14, 23]. The downfall of these PQ models was that they were not tested to ensure slow, progressive loss. However, progressive, continuous, and slow neuronal loss in the SNpc was tested and seen in our model during and after our 5 interpretational injection (1 every 5 days for 5 injections). Establishing this model is essential before testing any therapeutic treatment interventions as this is what characterizes PD in patients.

Additional research shows that the pathogenic mechanisms of PD are associated with mitochondrial dysfunction, oxidative stress and altered protein handling [4]. The involvement of mitochondria is considered a key to cell death observed in PD in both sporadic and familial cases.

Previous experiments in our lab have shown that Ubisol-Q_{10} is effective in protecting neurons against toxic insult *in vivo* and can protect DA neurons if administered prophylactically, that is, even before exposure to the environmental toxin, PQ [14]. However, PD is often not diagnosed until symptoms arise, which occurs when almost 50 – 60% neurons are lost.

Once the process is initiated by toxic insult, it is crucial to see if treatment administered therapeutically can halt further neurodegeneration. Ubisol-Q_{10} was tested therapeutically and it showed to have significant protection of the remaining DA neurons after both 4 weeks, and 8 weeks of treatment. This is one of the first experiments to show this. There are multiple explanations which could explain how Ubisol-Q_{10} protects the remaining neurons; initially it is

plausible that the combined anti-oxidant nature of the two components of Ubisol-Q_{10} (CoQ_{10} and Vitamin E) could quench the levels of oxidative stress associated with the disease. It was shown that the carrier solution containing vitamin E alone did not have a significant effect on neuroprotection ([14] and data not shown). In other research, it's been shown that lipid soluble CoQ_{10} (in high dosages) is an effective neuroprotective agent [19]. Past research on Ubisol-Q_{10} has shown it to be effective in stabilizing the mitochondria through inhibiting Bax [24]. Another hypothesis is that it could be protecting the mitochondria by increasing its overall energy output; as CoQ_{10} is naturally found in the electron transport chain.

Previous research using oil soluble CoQ_{10} as a treatment for PD made it into clinical trials in 2011, though failed in phase 2. In their pre-clinical work the oil-soluble CoQ_{10} treatment was tested prophylactically on MPTP induced mouse model [19,25]. The oil soluble CoQ_{10} was shown to be effective, but only at high dosages. A possible explanation to the discontinuation of their clinical trial was because very large dosages (1,600 mg/kg/day) were required to show any neuroprotection. When this dosage is converted to a human dose (averaging 70 kg) they are required to take 112 g/day in order to obtain results, which is beyond the acceptable FDA approved dose for clinical trials (2.4 g). Therefore, in the clinical trial they were not receiving anywhere near the dose required to show positive results. However, our preclinical work, on our more accurate chronological model, treating both prophylactically and therapeutically has shown comparable neuroprotection but at a significantly lower dose (6 mg/kg/day). Therefore, if our dosage was converted for human treatment it would only be 0.42 g/day, which is not only lower then FDA approved amount for clinical trial (2.4 g) but also the approved maximum daily dosage for general supplement intake (1.2 g). Both the oil soluble CoQ_{10} and Ubisol-Q_{10} showed

comparable bioavailability when administered, but in order to have comparable quantities in the brain the oil formulation needed to be given in a significantly higher dose [26].

The question remains why Ubisol-Q_{10} is more effective at lower doses than CoQ_{10}. It is assumed that the water soluble composition makes absorption into the blood stream easier, therefore, making it possible to cross the blood brain barrier. It is evident in our bioavailability experiment that this formulation does shuttle CoQ_{10} into the brain, due to the increase of 35% after 3 hours. Though, the other significant finding was that once it is in the brain it does not accumulate. This means that there is no build-up of CoQ_{10} in the brain, which could be toxic to the neurons. The natural removal seen explains why when the treatment is withdrawn the effects are no longer sustained. Henceforth, in order to sustain neuroprotection the treatment must be continuous and in doing so neurotoxicity will not result. It is also important to note that the animals in this experiment were allowed to drink Ubisol-Q_{10} supplemented drinking water ad libitum and were not gavaged.

Our study has shown that the withdrawal of Ubisol-Q_{10} leads to continued neurodegeneration, which was triggered by the toxin during the injection period. Therefore, Ubisol-Q_{10} does not halt neurodegeneration by acting on the toxin, but rather by supporting the remaining neurons. This experiment was only conducted with sustained treatment over 8 weeks (with 1 month of treatment and a consecutive month of withdrawal). To ensure the results, more research needs to be conducted over longer time spans. Though the current data found supports sustained treatment regiments in order to withstand neurodegeneration. These findings were also supported by the behaviour data which shows that animals provided with Ubisol-Q_{10} treatment for a longer

duration perform better throughout in the beam test compared to the animals where the treatment was withdrawn.

2.6 Conclusion

In conclusion we have shown that the PQ rat model of PD we used in the study shows slow progressive loss of DA neurons and hence mimics what is seen in patients suffering from PD. Our formulation can prevent the death of the remaining neurons in our PD model when administered after the process of neurodegeneration has been triggered and hence could be an effective therapeutic at any stage of the disease. Also, we found that Ubisol-Q_{10} has to be given continuously and cannot be withdrawn in order to continue neuroprotection. Bioavailability studies have shown that even though this formulation is provided at a low dose in order to provide significant neuroprotection, CoQ_{10} does cross the blood brain barrier and there is an increase in the levels of CoQ_{10} in the brain following administration of Ubisol-Q_{10}. This formulation of CoQ_{10} is FDA-GRAS approved and preliminary toxicity results show that there is no overt toxicity even when the dose is increased to 10 times the required dose. Ubisol-Q_{10} is an effective neuroprotective agent that could be used effectively to halt the progression of Parkinson's disease at low doses.

Authors' contributions

Krithika Muthukumaran, Marianna Sikorska, Jagdeep K Sandhu, Jerome Cohen and Siyaram Pandey contributed to the planning and execution of the experiments and writing the manuscript. Krithika Muthukumaran, Samantha Leahy, Kate Harrison, Harvey Miller and Patricia Lanthier were involved in performing injection and feeding of different regiments, dissections, immunohistochemical analysis and biochemical analysis. Jerome Cohen, Corrine Keshan and Daniel Lopatin were involved in the design and execution of behavioural tests, analysis of rotorod results and animal care. Marianna Sikorska, Henryk Borowy-Borowski and Shelly

Weinstock prepared water-soluble CoQ_{10} and placebo formulations. All authors read and approved the final manuscript.

2.7 References:

1. de Lau LM, Schipper CM, Hofman A, Koudstaal PJ, Breteler MM: Prognosis of Parkinson Disease Risk of Dementia and Mortality: The Rotterdam Study. *Arch Neurol* 2005, 62(8):1265–1269.

2. Olanow CW, Lees A, Obeso J: Levodopa therapy for Parkinson's disease: Challenges and future prospects·le first published online. *Mov Disord* 2008, 23(S3):S495–S496.

3. Klein C, Schlossmacher MG: Parkinson disease, 10 years after its genetic revolution: Multiple clues to a complex disorder. *Neurology* 2007, 69(22):2093–2104.

4. Davie CA: A review of Parkinson's disease. *Br Med Bull* 2008, 86:109–127.

5. Langston JW, Ballard P, Tetrud JW, Irwin I: Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 1983, 219:979–983.

6. Riachi NJ, Arora PK, Sayre LM, Harik SI: Potent Neurotoxic Fluorinated 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Analogs as Potential Probes in Models of Parkinson Disease. *J Neurochem* 1988, 50(4):1319–1321.

7. Sundstorm ME, Stromberg I, Tsutsumi T, Olson L, Jonsson G: Studies on the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholamine neurons in C57BL/6 mice. Comparison with three other strains of mice. *Brain Res* 1987, 405:26–38.

8. Heikkila RE, Sonsalla PK: The MPTP-treated mouse as a model of Parkinsonism: how good is it? *Neurochem Int* 1992, 20(Suppl):299S–303S.

9. Gerecke KM, Jiao Y, Pani A, Pagala V, Smeyne RJ: Exercise protects against MPTP-induced neurotoxicity in mice. *Brain Res* 2010, 1341:72–83.

10. Berry C, la Vecchia C, Nicotera P: Paraquat and Parkinson's disease. *Cell Death Differ* 2010, 17:1115–1125.

11. Rappold P, Cui M, Chesser AS, Tibbett J, Grima JC, Duan L, Sen N, Javitch JA, Tieu K: Paraquat neurotoxicity is mediated by the dopamine transporter and organic cation transporter-3. *Proc Natl Acad Sci U S A* 2011, 108(51):20766–20771.

12. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC: Environmental risk factors and Parkinson's disease: a case–control study in Taiwan. *Neurology* 1997, 48(6):1583–1588.

13. Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz M: Well-water consumption and Parkinson's Disease in rural California. *Environ Health Perspect* 2009, 117(12):1912–1918.

14. Somayajulu-Niţu M, Sandhu J, Cohen J, Sikorska M, Sridhar T, Matei A, Borowy-Borowski H, Pandey S: Paraquat induces oxidative stress, neuronal loss in substantia nigra region and Parkinsonism in adult rats: Neuroprotection and amelioration of symptoms by water-soluble formulation of Coenzyme Q10. *BMC Neurosci* 2009, 10:88.

15. Spindler M, Beal MF, Henchcliffe C: Coenzyme Q10 effects in neurodegenerative disease. *Neuropsychiatr Dis Treat* 2009, 5:597–610.

16. Borowy-Borowski H, Sodja C, Docherty J, Walker PR, Sikorska M: Unique technology for solubilization and delivery of highly lipophilic bioactive molecules. *J Drug Target* 2004, 12:415–424.

17. Sikorska M, Borowy-Borowski H, Zurakowski B, Walker PR: Derivatised alpha-tocopherol as a CoQ10 carrier in a novel water-soluble formulation. *Biofactors* 2003, 18(1–4):173–183.

18. Somayajulu M, McCarthy S, Hung M, Sikorska M, Borowy-Borowski H, Pandey S: Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol Dis* 2005, 18:618–625.

19. Cleren C, Yang L, Lorenzo B, Calingasan NY, Schomer A, Sireci A, Wille EJ, Beal MF: Therapeutic effects of coenzyme Q_{10} (CoQ_{10}) and reduced CoQ_{10} in the MPTP model of Parkinsonism. *J Neurochem* 2008, 104(6):1613–1621.

20. Andrello N, Santos E, Araujo M, Lopes L: Rat's age versus human's age: what's the relationship? *Arq Bras Cir Dig* 2012, 25(1):49–51.

21. Fornai F, Schlüter OM, Lenzi, Gesi M, Ruffoli R, Ferrucci F, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Südhof TC: Parkinson-like syndrome induced by continuous MPTP infusion: Convergent roles of the ubiquitin-proteasome system and α-synuclein. *Proc Natl Acad Sci U S A* 2004, 102(9):3413–3418.

22. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR, Comyns K, Richards MB, Meng C, Priestley B,

Fernandez HH, Cambi F, Umbach DM, Blair A, Sandler DP, Langston JW: Rotenone, paraquat, and Parkinson's disease. *Environ Health Perspect* 2011, 119(6):866–872.

23. Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW, Gross RE: Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. *Neurobiol Dis* 2005, 20(2):360–371.

24. Naderi J, Somayajulu-Nitu M, Mukerji A, Sharda P, Sikorska M, Borowy-Borowski H, Antonsson B, Pandey S: Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis* 2006, 11(8):1359–1369.

25. Yang L, Calingasan NY, Wille EJ, Cormier K, Smith K, Ferrante RJ, Beal MF: Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases. *J Neurochem* 2009, 109(5):1427–1439.

26. Constantinescu R, McDermott MP, Dicenzo R, de Blieck EA, Hyson HC, Beal MF, Bednarczyk EM, Bogdanov M, Metakis LJ, Browne SE, Lorenzo BJ, Ravina B, Kieburtz K: A randomized study of the bioavailability of different formulations of coenzyme Q(10) (ubiquinone). *J Clin Pharmacol* 2007, 47(12):1580–1586.

27. Graves S, Sikorska M, Borowy-Borowski H, Ho RJ, Bui T, Woodhouse C: Analysis of coenzyme Q10 content in human plasma and other biological samples. *Methods Mol Biol* 1998, 108:353–365.

28. Tang PH, Miles MV, Miles L, Quinlan J, Wong B, Wenisch A, Bove K: Measurement of reduced and oxidized coenzyme Q9 and coenzyme Q10 levels in mouse tissues by HPLC with coulometric detection. *Clin Chim Acta* 2004, 341(1–2):173–184.

29. Souchet N, Laplante S: Seasonal and geographical variations of sterol composition in snow crab hepatopancreas and pelagic fish viscera from Eastern Quebec. *Comp Biochem Physiol B Biochem Mol Biol* 2007, 147(3):378–386.

30. Maswood N, Young J, Tilmont E, Zhang Z, Gash DM, Gerhardt GA, Grondin R, Roth GS, Mattison J, Lane MA, Carson RE, Cohen RM, Mouton PR, Quigley C, Mattson MP, Ingram K: Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in aprimate model of Parkinson's disease. *Proc Natl Acad Sci U S A* 2004, 101(52):18171–18176.

31. Long JM, Kalehua AN, Muth NJ, Hengemihle JM, Jucker M, Calhoun ME, Ingram DK, Mouton PR: Stereological estimation of total microglia number in mouse hippocampus. *J Neurosci Methods* 1998, 84(1–2):101–108.

Chapter 3

Genetic susceptibility model of Parkinson's disease resulting from exposure of DJ-1 deficient mice to MPTP: Evaluation of neuroprotection by Ubisol-Q_{10}

3.1 Synopsis:

In most cases, Parkinson's disease (PD) arises from a combination of environmental and genetic risk factors. Especially, the early onset of PD is linked to certain neurotoxins exposure (paraquat, rotenone, MPTP) and to genetic mutations causing the functional defects in a family of PARKIN and α-synuclein genes. At present neither the curative nor preventative therapies are available; hence, there is an urgent need to develop reliable animal models to facilitate their development. We have combined the genetic deficiency of DJ-1/PARK7 mice with MPTP exposure and develop a genetic susceptibility model of PD. Subsequently we evaluated the neuroprotective efficacy of water soluble formulation of CoQ_{10} (Ubisol-Q_{10}). CoQ_{10} has shown some promise in halting neurodegeneration in PD studies performed to date, its lipophilic nature limits the bioavailability. In this study, mice received Ubisol-Q_{10} prophylactically at a dose of 6 mg/kg/day added directly to a drinking water. The outcomes were evaluated based on stereological counts of surviving tyrosine hydroxylase-positive neurons in the SNpc region and the behavioral improvements. The data showed that the DJ-1 deficient mice were very sensitive to the MPTP toxicity and revealed that Ubisol-Q_{10} provided orally delivered a very significant neuroprotection, even at the dose several folds lower than previously tested. Thus, we showed for the first time, that the prophylactic treatment of DJ-1 deficient mice with Ubisol-Q_{10} prevented the degeneration of DA neurons triggered by MPTP. Our results encouragingly imply that a preventative therapy for people genetically predisposed to PD might be possible.

3.2 Introduction:

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders, affecting 1–2% of all individuals over the age of 60 and this number steadily increases as the population ages [1]. The clinical diagnosis is based on the decline in motor function bradykinesia, rigidity and postural instability), which stem from the loss of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) region of the brain [2]. These symptoms develop after 60–70% of the DA neurons are already eliminated [2], highlighting the urgent needs to develop more effective treatments. The disease etiology is still unknown and the majority of cases are considered sporadic. Over the years several environmental toxins (i.e., paraquat, MPTP, rotenone) are shown to selectively kill DA neurons in the brain. The widely used herbicide paraquat selectively induces death of the SNpc neurons in animal models and several studies indicate a greater risk of PD among subjects exposed to paraquat [3, 4]. The selective killing of DA neurons by MPTP remains one of the most commonly used PD models in research today. All these toxins are the inhibitors of complex I of the mitochondrial electron transport chain, causing neuronal death by ATP depletion and generation of oxidative stress [5]. Even under normal conditions, the DA neurons are exposed to high levels of oxidative stress due to the production of reactive oxygen species during dopamine metabolism [5]. The hypothesis for oxidative stress as a primary trigger of neuronal death has been supported by post-mortem studies of PD brains [6]. In the past decade over 18 genetic loci have been linked to the familial forms of PD revealing that approx. 10% of all PD cases exhibit Mendelian inheritance. Of the many genes implicated is PARK7/DJ-1. DJ-1 is a member of C56 family of peptidases, shown in vitro to protect neurons against oxidative stress and cell death due to its putative function as a redox sensitive chaperone and sensor for oxidative stress [7]. A homozygosity in DJ-1 loss-of-

function mutation accounts for 1-2% of early-onset of PD (EOPD) cases [8]. Although this loss-of-function mutation predisposes humans to EOPD, no nigrostriatal degeneration were found in DJ-1 deficient mice [9]. Therefore, an animal model recapitulating the characteristics of parkinsonian neurodegeneration needs to be developed in order to study this form of hereditary PD [10]. Here we described such a model by exposing the DJ-1 deficient transgenic mice to MPTP neurotoxicity. We used this model to test the utility of Ubisol-Q_{10} as a neuroprotectant. Coenzyme Q10 (CoQ_{10}) has been under investigation as a potential therapy for PD for over a decade. CoQ_{10} levels in the brain, and other tissues, decrease with age in human and animal tissues [11]. The SNpc region has the lowest CoQ_{10} content in the brain [12]. The highly hydrophobic CoQ_{10} in oil-soluble formulations has not been successful in clinical trials, and the preclinical efficacy for neuroprotection requires extremely high doses [13, 14]. An apparently water-soluble nanomicellar formulation of CoQ_{10}, Ubisol-Q_{10}, was developed by the National Research Council of Canada (US Patent No. 6,045,826) by exploiting the self-emulsifying properties of polyoxyethanyl-tocopheryl sebacate (PTS). Ubisol-Q_{10} showed significantly enhanced bioavailability and, consequently, neuroprotection of the SNpc neurons when given either prophylactically or therapeutically at low doses to rats exposed to paraquat and mice treated with MPTP [3, 15, 16]. In the present study we evaluated neuroprotective efficacy of Ubisol Q_{10} in DJ-1/MPTP model of PD using histochemical and behavioral read outs. We confirmed genetic susceptibility to MPTP and showed that prophylactic oral treatment with Ubisol-Q_{10} significantly offset the neurotoxicity and ameliorated motor dysfunction, otherwise correlated with the MPTP injury. These results offer some hope for finding a preventative treatment for humans genetically predisposed to PD.

2.3 Materials and methods:

Animal care and experimental treatments

Male DJ-1 deficient and C57BL/6 wild type mice (Jackson Laboratory) were divided into four groups: (i) control (saline- injected and drinking regularwater); (ii) unprotected (MPTP-injected and drinking regular water); (iii) protected (MPTP-injected and drinking Ubisol-Q10 supplemented water containing 50 g/ml of CoQ_{10} and 150 g of PTS/ml); (iv) placebo–PTS (MPTP-injected and drinking PTS supplemented water containing 150 g PTS/ml). Ubisol-Q_{10} and PTS were provided by Zymes LLC (Hasbrouck, NJ). The MPTP injected mice received intraperitoneal (i.p.) injections of MPTP at 20 mg/kg once a day for six consecutive days [17]. Control group was injected with saline in a similar way. Animals were housed under standard conditions: constant temperature of 20°C, 12 hour dark-light cycle and free access to food and drinking solutions. The experiments were carried out for total of 8 weeks with Ubisol-Q_{10} and PTS supplementations starting 4 weeks prior to the MPTP injection and continued until the final evaluations 4 weeks after the last injection. All procedures and protocols were approved by the University of Windsor Animal Care Committee (AUPP#11-07) and were carried out in accordance with the EU Directive 2010/63/EU.

Immunohistochemistry and stereological data analysis

The mice were anesthetized and perfused with a minimum of 10 mL of Tyrodes solution containing buffered 10% formaldehyde and stored at 4°C as previously described [15]. Coronal midbrain sections at 30 m were cut on a Leica CM3050S cryostat and were immunostained with anti-tyrosine hydroxylase (TH) antibody as described before [3, 16]. The TH positive neurons in

the SNpc were counted (on one side) in every fourth brain section (total 12 sections per brain) using a Stereologer 2000 software (Stereology Resource Center Inc., Chester, MD) as described previously [15]. Statistical significance of the data was calculated using GraphPad Prism 5.

Analysis of tissue MPP+ contents

Mice received either regular drinking water or Ubisol - Q_{10} supplemented drinking water (30 mg CoQ_{10}/kg body weight, ad libitum) for 2 weeks and then were challenged with a single intraperitoneal injection of MPTP (25 mg/kg body weight). They were sacrificed either 90 minutes or 4 hours post-MPTP injection. Tissues, striatum and liver, were collected and analyzed for the content of MPP+ using the HPLC method [16]. Briefly, tissues were homomogenized in 10 volumes of ice-cold 0.1 M perchloric acid and 0.1mM EDTA containing 10 µM 4-phenylpiridine (Sigma-Aldrich, Oakville) as an internal standard. Clear supernatants were injected onto a reverse phase C18 HPLC column (4.6×150 mm; TSK-GEL ODS-100 S, 7_ particle size; Tosoh Biosep LLC, Montgomereyville) equipped with a 1 mm C18 OPTI-GUARD column (Optimize Technologies, Oregon City, OR). The mobile phase, consisting of 0.02 M NaH_2PO_4, 3mM tetrabutylammonium bisulfate (Sigma-Aldrich), 0.5mM 1-heptanesulfonic acid sodium salt (Sigma-Aldrich) and 10% isopropanol adjusted to pH 2.5 with orthophosphoric acid, was delivered at a flow rate of 1.0 mL/min at ambient temperature. Eluting peaks were detected by UV at 293 nm (System Gold® HPLC, model 166 Programmable UV Detector Module; Beckman-Coulter Canada Inc., Mississauga, ON) and were analyzed using Beckman- Coulter's System Gold 32 Karat™ software. The data is reported as nmoles of MPP+ per g of tissue and is plotted using GraphPad Prism 6.0.

Horizontal beam test: Behavioral assessment

All mice were assessed for performance on a horizontal beam-walking test for motor skills deficits by measuring the leg slips according to the previously described protocol [18]. The animal had to traverse a 1.03 m long and 6 mm by 20 mm aluminum beam to enter a 'safe' 20 cm^3 black chamber. This apparatus was placed 0.5 m above a rectangular box containing saw dust to cushion any fall or prevent any escape. A mirror on the wall extended along the length of the beam allowed an unobstructed view of the animal as it moved towards the covered chamber. The locomotor activities of each mouse were recorded with a standard digital video camera (JVC) located 2.15 m away from the center of beam and 0.23 m above it. Video clips for each beam test session were recorded and the numbers of leg slips were counted. These data were analyzed by comparing least significant differences and by multiple post-hoc comparisons. Effects were considered significant at $p \leq 0.05$.

2.4 Results and discussion:

Effects of MPTP and Ubisol-Q10 on DJ-1 deficient mice

Mice were given 6 daily injections of MPTP and the surviving TH+ neurons in the SNpc region were counted 4 weeks after the last injection. This termination time point was selected based on the recent study showing that in mice subjected to this sub-chronic MPTP model the neuronal death processes are completed by 4 weeks post the last MPTP injection [16]. Consistent with the later study, we have established here that following such a sub-chronic exposure to MPTP, approx. 50% of the SNpc DA neurons in the DJ-1 deficient mice (Fig. 8B) and approx. 44% in the wild type mice (Fig. 9B) were lost at 4 weeks post-treatment. This was clearly evident on TH-stained photomicrographs of the tissue sections (Figs. 8A and 9A) as well on stereological counts of TH-positive cells (Figs. 8B and 9B). Although, the difference in cell loss between these two strains of mice was not very big, it confirmed previously published work [17] indicating that DJ-1 deficiency rendered these mice more sensitive to the MPTP toxicity. We have previously reported that Ubisol-Q_{10} protects DA neurons against paraquat toxicity and prevents degeneration of SN in rat model of PD [3]. Subsequently, we have established that Ubisol-Q_{10} acts as very effective neuroprotectant when given therapeutically, i.e., delivered post-neurotoxin exposure as a supplement in drinking water [15, 16]. This is true for both paraquat in rats and MPTP in mice and it is effective for as long as the supplementation continues. Upon discontinuation of the supplementation the neurodegeneration commences again and neuronal cell death processes are reactivated [15, 16]. In the present study we addressed a question whether this formulation could prevent neurodegeneration in genetically predisposed individuals where the link between mutation in the gene and the risk of developing PD has already been

established. Therefore, we tested its efficacy in a genetic susceptibility model of PD. In these experiments mice were given prophylactically Ubisol-Q_{10} supplemented drinking water, starting 4 weeks prior to the MPTP injections and continued for 4 more weeks after the last injection (Figs. 8 and 9). The results revealed a significant neuroprotection by Ubisol-Q_{10} in the MPTP-treated DJ-1 mice, with less than 20% of DA neuronal loss in the protected group (MPTP/Ubisol-Q_{10}) as compared to nearly 50% in the unprotected (MPTP/H_2O) and 40% in the placebo (MPTP/PTS) groups (Fig. 8). Even more robust neuroprotection by Ubisol-Q_{10} was found in the wild type mice (Fig. 9). Here, in the protected group (MPTP/Ubisol-Q_{10}) only a negligible percentage of DA neurons were lost (approx.6%) in comparison to 44% in the unprotected group (MPTP/H_2O). In these experiments, mice received on average 6mg CoQ_{10}/kg/day, similar to the concentrations previously used in rats and mice [15, 16]. In the later studies we show that the neuroprotection correlated with the brain penetration of CoQ_{10} [15, 16]. We have also ruled out a possibility that this neuroprotection was due to an interaction between CoQ_{10} and MPTP preventing the generation of the neurotoxic MPP+. As shown in Fig. 10, the same levels of MPP+ were measured in tissues of control mice and mice drinking for 2 weeks Ubisol-Q_{10} supplemented water, even when Ubisol-Q_{10} was applied at a concentration 5 times higher (30 mg/kg CoQ_{10}) than that used to achieve the described above neuroprotection (6 mg/kg). The data revealed higher contents of MPP+ at 90 minutes than at 4 hours post-injection indicating further its unaffected metabolism and showing that the presence of CoQ_{10} did not interfere with the brain penetration of MPP+. The neuroprotective effectiveness of Ubisol-Q_{10} at such a low dose is in a sharp contrast with previous preclinical data obtained on the mouse MPTP model using an oil-soluble formulation 'Tishcon CoQ_{10}'[14].

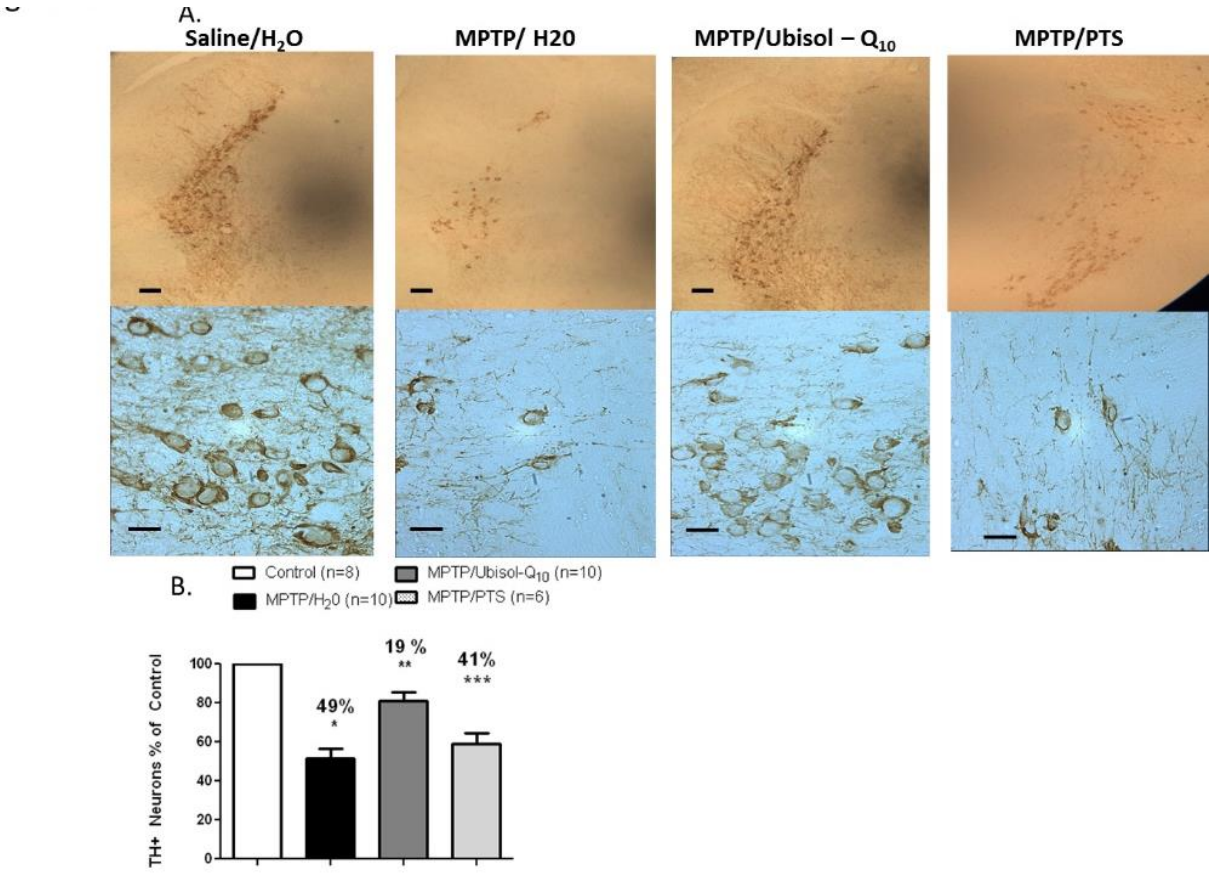


Figure 8 Effects of Ubisol-Q_{10} on the survival of TH-positive neurons in the SNpc of MPTP-treated DJ-1 deficient mice. Four weeks prior to the MPTP injections mice were given regular drinking water, water supplemented with Ubisol-Q_{10} or with PTS. Subsequently they were injected with either MPTP or saline (6 daily injections). The supplementations with Ubisol-Q_{10} and PTS continued for the additional 4 weeks until the termination of the experiments. Four groups of mice were examined: (i) saline/H_20 (saline injected and drinking regular water, control); (ii) MPTP/H_2O (MPTP injected drinking regular water, unprotected); (iii) MPTP/Ubisol-Q_{10} (MPTP injected receiving Ubisol-Q_{10} supplementation, protected) and MPTP/PTS (MPTP injected receiving PTS placebo). (A) Representative photomicrographs of anti-tyrosine hydroxylase stained brain sections taken at lower and higher magnifications and showing normal distribution of TH-positive neurons in control brains (saline/H_20), significantly reduced TH immunostaining reflection the loss of DA neurons in unprotected brains (MPTP/H_20) and preservation of TH-positive cell bodies and neuronal fibres in the Ubisol–Q_{10} protected brains (MPTP/Ubisol-Q_{10}). All mice were dissected one month after the last injection. The bars are: 200 µm in the upper (low magnification) panel and 20 µm in the lower (high magnification) panel. (B) Survival of TH-positive neurons in the SNpc calculated using the Stereologer 2000 software. Every 4th section of the midbrain (sectioned at 30 microns thickness) was analysed, in total 12 sections per each mice. The number of TH positive neurons in each experimental group of animals was established and the results are expressed as percentage of neurons found in the SNpc of control brains (saline/H_20). The data showed 49% decline in the

number of TH - positive neurons in the MPTP/H_20 group (* $p<0.05$ in comparison to saline/H_20, unprotected vs. control), 41% in MPTP/PTS group (***$p<0.05$ in comparison to saline/H_20, placebo vs. control), but only 19% in the MPTP/Ubisol-Q_{10} (**$p< 0.05$ in comparison to MPTP/H_20, protected vs. unprotected).

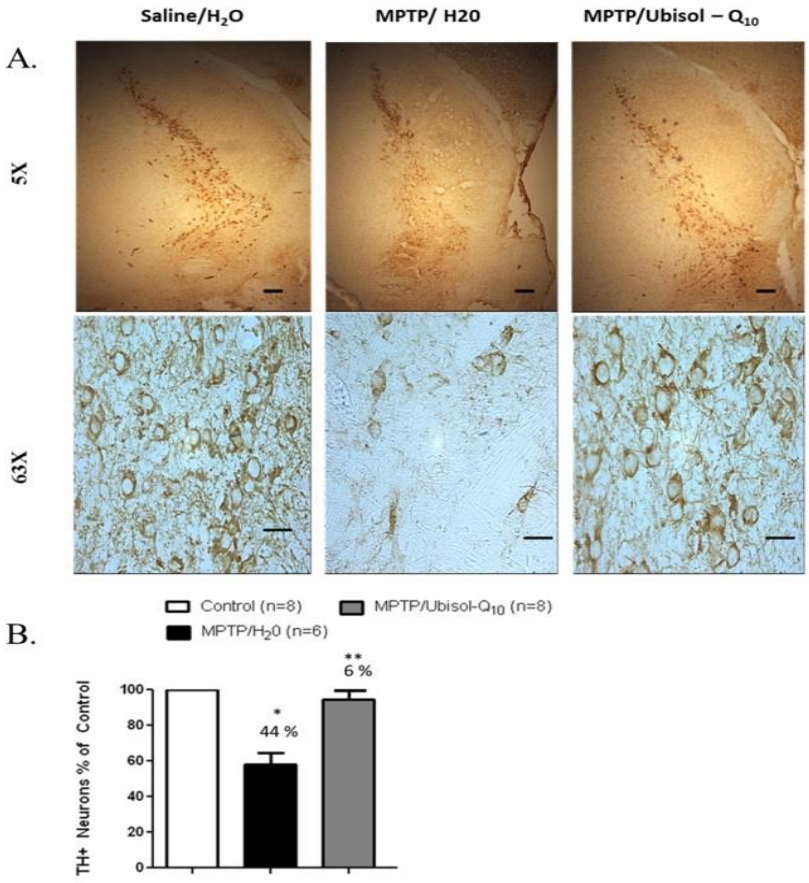
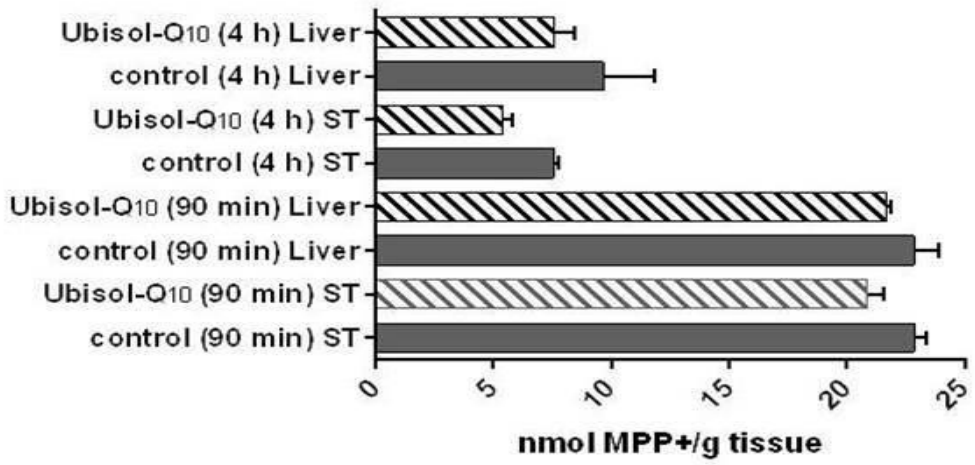


Figure 9 Effects of Ubisol-Q_{10} on the survival of TH-positive neurons in the SNpc of MPTP-treated wild type mice. The experimental treatments of the wild type mice (i.e., Ubisol-Q_{10} supplementation, MPTP injections) were the same as the DJ-1 deficient transgene. Three groups of mice were examined: (i) saline/H_20 (saline injected and drinking regular water, control); (ii) MPTP/H_2O (MPTP injected drinking regular water, unprotected); (iii) MPTP/Ubisol-Q_{10} (MPTP injected receiving Ubisol-Q_{10} supplementation, protected). (A) Representative photomicrographs of anti-tyrosine hydroxylase stained brain sections showing normal distribution of TH-positive neurons in control brains of wild type mice (saline/H_20), reduced TH-immunostaining and the loss of DA neurons in unprotected brains (MPTP/H_20) and the preservation of TH-positive cell bodies and neuronal fibers in the Ubisol –Q_{10} protected brains (MPTP/Ubisol-Q_{10}). All mice were dissected one month after the last injection. The bars are: 200 µm in the upper (low magnification) panel and 20 µm in the lower (high magnification) panel. (B) Survival of TH-positive neurons in the SNpc calculated using the Stereologer 2000 software. Every 4th section of the midbrain (sectioned at 30 microns thickness) was analysed, in total 12 sections per each mice. The number of TH positive neurons in each experimental group was established and the results are expressed as percentage of neurons found in the SNpc of control brains (saline/H_20).

There was a significant decrease in the number of TH - positive neurons in the unprotected group (MPTP/$H_2$0) as compared to the saline injected control group (44% cell loss ; * $p<0.05$, unprotected vs. control). The Ubisol-Q_{10} supplementation brought about nearly complete neuroprotection (only 6% neurons were lost; ** $p<0.05$, protected vs. unprotected).

The reported neuroprotection required extremely high doses of CoQ_{10}, i.e., 400–1600 mg/kg/day, which could not be applicable in the patients' care. On the other hand, the effective Ubisol-Q_{10} dose would translate to 420 mg CoQ_{10} per day for a 70 kg patient signifying the need for its further clinical evaluation. Although the mechanism of neuroprotection by Ubisol-Q_{10} is not fully understood, evidence points toward its role in mitochondrial stabilization. The DJ-1 deficient mice have been shown to display mitochondrial dysfunction and defect in anti-oxidative defense mechanism [7, 9]. Ubisol-Q_{10} contains, potentially, two potent antioxidants CoQ_{10} and a pro-drug form of α-tocopherol (PTS). However, tested alone PTS did not produce any neuroprotection against MPTP (Fig. 8 and 10) suggesting that the observed effects could mainly be ascribed to the properties of CoQ_{10} delivered as Ubisol-Q_{10}. In already published *in vitro* studies Ubisol-Q_{10} prevents oxidative stress-induced neuronal cell death and inhibits Bax-induced mitochondrial destabilization [19, 20]. Ubisol-Q_{10} could very well maintain the integrity of mitochondria and quench ROS produced in the cells. Ubisol-Q_{10} might be able to compensate to some extent for the deficiency of DJ-1 function as it relates to the mitochondrial integrity and anti-oxidative defense mechanisms.



ANOVA, P<0.0001- GraphPad Prism 6.0

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
control (90 min) ST vs. WS-CoQ10 (90 min) ST	1.985	-1.102 to 5.072	No	ns
control (90 min) Liver vs. WS-CoQ10 (90 min) Liver	1.140	-1.947 to 4.227	No	ns
control (4 h) ST vs. WS-CoQ10 (4 h) ST	2.165	-0.9219 to 5.252	No	ns
control (4 h) Liver vs. WS-CoQ10 (4 h) Liver	2.040	-1.047 to 5.127	No	ns

Figure 10 MPP+ content in the liver and brain tissues. Experimental mice were placed on Ubisol-Q10 supplemented water (30 mg CoQ10/kg body weight) and control mice on regular water for 2 weeks prior to a single intra-peritoneal injection of MPTP at 25 mg/kg. Brain (striatum –ST) and liver tissues were collected at 90 min and 4 h after the MPTP injection. MPP+ levels were measured by HPLC as described in Materials and Methods. Data is represented as mean±SD (n = 3 per group, from a single experiment). No statistically significant differences were observed between the groups ($P \geq 0.05$, MPTP ST vs MPTP/Ubisol-Q_{10} ST at 90 min and 4 h; $P \geq 0.05$, MPTP liver vs MPTP/Ubisol-Q_{10} liver at 90 min and 4 h).

Assessment of post-injection behavioral disruption in beam walking

As an additional measure of Ubisol-Q_{10} neuroprotection, we applied a beam walk test to examine the animals' motor skills [18]. We used the leg slip data from the last pre-injection beam test session as a baseline and compared it to the performance on the three post-injection tests. As shown in Fig. 11, the DJ-1 deficient mice of the unprotected group (MPTP/water) displayed a higher number of leg slips on each of the three post-injection beam tests than the mice in saline injected control group or the protected MPTP/Ubisol-Q_{10} group, neither of which showed any significant changes from their respective baseline. These observations were confirmed by a significant interaction between groups and sessions, $F6,57 = 2.532$, $p = 0.03$. This interaction resulted from a significant effect for sessions involving only the MPTP/water group, $F3,21 = 6.893$, $p = 0.002$, suggesting that these animals significantly increased their post-injection leg slips above their last pre-injection session level. The performance of DJ-1 deficient mice on the beam test correlated with the MPTP-induced loss of DA neurons. In summary, we have developed a genetic susceptibility model of PD by combining the DJ-1/PARK 7 defects with the systemic exposure to MPTP. We confirmed the hypersensitivity of DJ-1 transgenic animals to MPTP and demonstrated a clear neuroprotection by a prophylactic use of Ubisol-Q_{10}. Since Ubisol-Q_{10} is already GRAS approved by the FDA, it represents a promising and realistic prospect of preventative therapy for people genetically predisposed to PD.

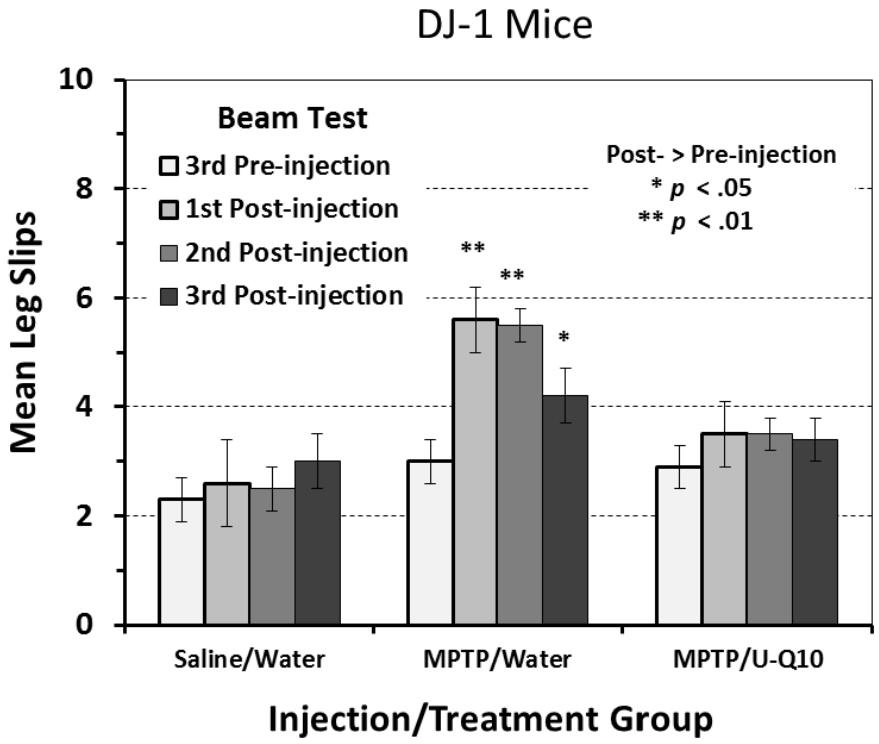


Figure 11 Behavioral improvements. The experimental treatments (Ubisol supplementation, MPTP injections) of DJ-1mice were the same as described in Fig.1. Three groups of mice were tested: (i) control -saline/H_20 (saline injected and drinking regular water); (ii) unprotected -MPTP/H_2O (MPTP injected drinking regular water) and, (iii) protected -MPTP/Ubisol-Q_{10} (MPTP injected receiving Ubisol-Q_{10} supplementation). Mice received pre-test beam training session followed by three widely distributed test sessions prior to the MPTP injections (pre-injection beam tests) executed on the 2nd, 9th, and 16th day from the start of the experiment and three similarly widely distributed test sessions (post-injection beam tests) starting two days after the last injection day and repeated on days 9 and 16 post-injection. During the pre-test training session, the mice were first placed for 2 min into the end chamber and then positioned on the beam at the increasing distances of 0.25 m, 0.5 m and 1 m from the end chamber to which they had to run. On each of the six beam test sessions, the mice were placed at the end of the beam facing the black box and had up to two minutes to run into the end chamber. A mouse, which did not complete this session within two minutes was removed from the beam and returned to its home cage. Each beam test session was recorded and the numbers of leg slips were counted. The data is plotted as the mean number of leg slips on the horizontal beam made by each group of DJ-1 deficient mice during the third pre-injection test (baseline) and each of the three post-injection tests. The vertical error bars represent $\pm$SEM. Of the three experimental groups tested only the unprotected MPTP /H_20 group showed a significantly greater number of leg slips during the first and second (** $p<.01$) or third (*$p<.05$) post-injection tests in comparison to baseline (the third pre-injection test).

Author's contribution:

Krithika Muthukumaran, Jessica Smith, Marianna Sikorska, Jagdeep K Sandhu, Jerome Cohen and Siyaram Pandey contributed to the planning and execution of the experiments and writing the manuscript. Krithika Muthukumaran, Jessica Smith and Harshil Jasra were involved in performing injection and feeding of different regiments, dissections, immunohistochemical analysis and biochemical analysis. Jerome Cohen, Daniel Lopatin, Krithika Muthukumaran and Jessica Smith were involved in the design and execution of the beam test and animal care.

2.5 References:

1. Gasser T (2009) Mendelian forms of Parkinson's disease. Biochim Biophys Acta., 1792(7):587-96.
2. Lang AE, Lozano AM (1998) Parkinson's disease-Second of two parts. N Engl J Med, 339(16):1130-1143.
3. Somayajulu-Nițu M, Sandhu JK, Cohen J, Sikorska M, Sridhar TS, Matei A et al (2009) Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of coenzyme Q10. BMC Neurosci, 10:88.
4. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY et al (1997) Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology, 48(6):1583-8.
5. Dexter DT, Sian J, Rose S, Hindmarsh JG, Mann VM, Cooper JM et al., (1994) Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. Ann Neurol, 35(1):38-44.
6. Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol, 53:S26-S36.
7. Trancikova A, Tsika E, Moore DJ (2012) Mitochondrial dysfunction in genetic animal models of Parkinson's disease. Antioxid Redox Signal, 16(9):896-919.
8. van Duijn CM, Dekker MC, Bonifati V, Galjaard RJ, Houwing-Duistermaat JJ, Snijders PJ et al. (2001) Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. Am J Hum Genet, 69(3):629-634.
9. Andres-Mateos E, Perier C, Zhang L, Blanchard-Fillion B, Greco TM, Thomas B, Ko HS, Sasaki M, Ischiropoulos H, Przedborski S, Dawson TM, and Dawson VL (2007) DJ-

1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. Proc Natl Acad Sci USA, 104: 14807–14812.

10. Chandran JS, Lin X, Zapata A, Höke A, Shimoji M, Moore SO et al. (2008) Progressive behavioral deficits in DJ-1 deficient mice are associated with normal nigrostriatal function. Neurobiol Dis, 29(3):505-14.
11. Tieu K (2011) A guide to neurotoxic animal models of Parkinson's disease. Cold Spring Harb Perspect Med., 1(1): 009316.4.
12. Sharma S, Kheradpezhou M, Shavali S, El Refaey H, Eken J, Hagen C et al. (2004) Neuroprotective actions of coenzyme Q10 in Parkinson's disease. Methods Enzymol, 382:488-509.
13. Strijks E, Kremer HP, Horstink MW (1997) Q10 therapy in patients with idiopathic Parkinson's disease. Mol Aspects Med., 18(Suppl):S237–S240.
14. Cleren C, Yang L, Lorenzo B, Calingasan NY, Schomer A, Sireci A et al. (2008) Therapeutic effects of coenzyme Q(10) (CoQ(10)) and reduced CoQ(10) in the MPTP model of Parkinsonism. J Neurochem, 104(6):1613–1621.
15. Muthukumaran K, Leahy S, Harrison K, Sikorska M, Sandhu JK, Cohen J, Keshan C, Lopatin D, Miller H, Borowy-Borowski H, Lanthier P, Wienstock S, Pandey S. (2014) Orally delivered water soluble Coenzyme Q10 (Ubisol-Q10) blocks on-going neurodegeneration in rats exposed to paraquat: potential for therapeutic application in Parkinson's disease. BMC Neurosci, 14:21.
16. Sikorska M, Lanthier P, Miller H, Beyers M, Sodja C, Zurakowski B, Gangaraju S, Pandey S, Sandhu JK (2014) Nanomicelar formulation of Coenzyme Q10 (Ubisol-Q10)

effectively blocks ongoing neurodegeneration in the mouse MPTP model: potential use as an adjuvant treatment in PD .

17. Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP, Pownall S et al. (2005) Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) and oxidative stress. Proc Natl Acad U S A, 102(14): 5215-20.
18. Carter RJ, Morton J, Dunnett SB (2001) Motor coordination and balance in rodents. Curr Protoc Neurosci Unit, 8 - 12.
19. Somayajulu M, McCarthy S, Hung M, Sikorska M, Borowy-Borowski H, Pandey S (2005) Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q(10). Neurobiol Dis, 18(3):618-627.
20. Naderi J, Somayajulu-Nitu M, Mukerji A, Sharda P, Sikorska M, Borowy-Borowski H et al. (2006) Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. Apoptosis, 11(8):1359-1369.

Chapter 4

Inhibition of amyloid plaque formation and memory deficits by Ubisol-Q_{10} (nanomicelle formulation of CoQ_{10}) in a double transgenic mouse model of Alzheimer's disease

4.1 Synopsis:

Alzheimer's disease (AD) is a progressive, fatal neurodegenerative disease affecting over 500,000 Canadians. AD is characterized by amyloid-β plaques and neurofibrillary tangles in association with neuronal death. Research has pointed to oxidative stress as a mechanism of neurodegeneration that leads to cognitive deficits, and impaired behaviour. Using antioxidants to combat free-radical damage and decrease oxidative stress has become an area of interest. A water-soluble formulation of Coenzyme Q_{10} (Ubisol-Q_{10}) has shown to provide neuroprotection in animal models of Parkinson's disease. Interestingly, *in vitro* studies using Ubisol-Q_{10} prevented stress induced premature senescence in AD fibroblasts. Therefore, we investigated if this formulation could prevent the progression of AD in a genetically predisposed double transgenic mouse model of AD, expressing human amyloid precursor protein and mutant human presenilin-1 genes. The treatment continued for 18 months whereby the treated group received Ubisol-Q_{10}-supplemented water and the untreated group received regular water. Behavioural studies indicated improved long-term memory and emotional reactivity in the treated transgenic group. Immunohistochemistry and Congo red staining both showed a decrease in the level of amyloid plaque in treated transgenic mice. Further, immunohistochemistry analysis revealed an increase in astrocyte activation, but a decrease in microglial activation in the treatment groups. Preliminary results indicated neurogenesis in the hippocampus. Thus, Ubisol-Q_{10} has

demonstrated a potential in preventing one of the key hallmarks in Alzheimer's disease in addition to preventing memory deficits.

4.2 Introduction:

Alzheimer's disease (AD), the most common form of dementia, is characterised by progressive decline in cognitive function and is a major cause of morbidity and mortality [1]. Since age is the greatest risk factor for this disease, an ageing population poses a serious medical and economic crisis in the coming decades. The key pathological features associated with AD include; 1) loss of neurons in the hippocampus region leading to learning and memory impairments, 2) aggregation and deposition of amyloid fibrils, forming neuritic plaques outside neurons, and 3) accumulation of hyperphosphorylated tau proteins, which form neurofibirillary tangles (NFTs) inside the nerve cell bodies. [2]. The majority of the AD cases are sporadic, however, mutations in genes linked to early onset and familial cases of AD have also been identified, contributing to 10% of reported AD cases. Mutations in genes involved in the encoding of amyloid beta (Aβ), presenilin 1 (PS1) and presenilin 2 (PS2) have been linked to early onset autosomal dominant AD. Both PS1 and PS2 are part of γ- secretase enzyme that cleaves amyloid precursor protein (AβPP) via the β-secretase pathway to give rise to Aβ [3,4,5]. The FDA approved treatments currently available are acetylcholine esterase inhibitors and NMDA receptor antagonists which can only stabilise the symptoms for a short period in early to moderate stages of the disease [6,7]. This calls for extensive research and development of new approaches to halt disease progression in order to face the challenge posed by the rising ageing population.

Two of the important hallmarks of ageing implicated in a number of ageing related diseases such as AD are impaired ubiquitin proteasome system (UPS) and mitochondrial dysfunction [8]. The UPS along with autophagy is involved in clearing the unwanted damaged proteins that could become toxic to the cell [9, 10]. UPS and autophagy are two critical carefully orchestrated pathways that act in synergy and degrade majority of the proteins in eukaryotes [10].

Mitochondria, which generate energy via oxidative phosphorylation, is the largest source of reactive oxygen species (ROS), and uncontrolled ROS can damage proteins, lipids and DNA. An increase in the levels of ROS means an increase in the damaged proteins that have to be removed [11]. Therefore, targeting mitochondrial dysfunction, could potentially help protect and prevent the consequences of ageing.

For a very long time the focus of AD pathophysiology has been on Aβ plaques and the NFTs, however, evidence indicates that mitochondrial dysfunction and an increase in oxidative stress has a bigger role in AD and is responsible for abnormalities in synapses and neurodegeneration [12,13]. Proteomics studies have also shown that the defects in the mitochondria are predominantly in complex I, IV and V of the electron transport chain (ETC) [14]. This leads to the generation of free radicals. Hence, one of the methods to approach treatment of AD would be to restore mitochondrial function, reduce the levels of oxidative stress and quench the free radicals by using an antioxidant. One such naturally occurring, coincidental antioxidant is Coenzyme Q_{10} (CoQ_{10}), which is part of the ETC and has been extensively studied and tested on animal models of neurodegenerative disorders [15].

Despite CoQ_{10} showing effective neuroprotection in animal models of neurodegenerative diseases, it has failed to show promise in clinical trials of Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis [16,17,18]. This is due to poor bioavailability. In a rat model, the effective dose of the oil soluble formulation required to provide significant neuroprotection is 200-1600 mg/kg/day, which translates to a very high dose, well above the FDA approved dose for humans, hence failing to show potential as a treatment in clinical trials.

Attempts ave been made to improve the bioavailability of CoQ_{10}. One such formulation of CoQ_{10} is Ubisol-Q_{10}, which was developed by the National Research Council (NRC) in Ottawa. This

formulation is water soluble and the carrier used for solubilisation of CoQ_{10} consists of polyethylene glycol linked to α-tocopherol via an alkanedioyl linker, sebacic acid, giving rise to the stable non-toxic Ubisol-Q_{10} [19]. Previous studies in our lab have shown that Ubisol-Q_{10} is able to provide successful neuroprotection by halting neurodegeneration in both an environmental toxin model and a genetic susceptibility model of Parkinson's disease. The bioavailability is much improved with this formulation; the effective dose required in rodent models is 6 mg/Kg/day [20, 21]. In addition, Ubisol-Q_{10} is able to decrease the levels of reactive oxygen species generated by fibroblasts of AD patients with mutations in the PS-1 gene, stabilise the mitochondria, and delay the onset of premature senescence in vitro [22, 23]. We have now tested the effects of prophylactic treatment with Ubisol-Q_{10} in a mouse model expressing chimeric human/mouse amyloid precursor protein (AβPP) and mutant human PS1. These transgenic mice secrete elevated levels of human Aβ peptide due to the presence of Swedish mutations that favour processing of AβPP via the β-secretase pathway. The efficacy of Ubisol-Q_{10} was evaluated in this pilot study by studying the changes in the pathological features and behavioural symptoms with treatment.

As the focus is slowly being shifted from neurons being the fundamental unit of the brain towards the neuron-microglia-astrocyte triad, the contribution of astrocytes and microglia in the pathophysiology of AD is becoming increasingly recognised. Studies suggest the presence of reactive astrocytes and primed microglia in the hippocampus region especially at the vicinity of the Aβ plaques. Hence, changes in astrocytes and microglia morphology in the transgenic mice provided with Ubisol-Q_{10} treatment was studied as well.

We report the ability of prophylactic treatment with Ubisol-Q_{10} to reduce the extent of plaque deposits and cognitive deficits that are observed in these transgenic mice and also induce changes in the glial cells morphology.

4.3 Materials and Methods:

Animal care

Experiments on the animals were conducted following approval from the Animal Care Committee at the University of Windsor. All the guidelines of the Canadian Council for Animal care were followed. One month old double transgenic male mice containing human/mouse chimeric AβPP and a mutant PS1 gene and their wild type counterparts were purchased from MMRRC. Mice were allowed continuous access to food and water in their group cages. The room the mice were housed in was maintained at 20 ˚C and reversed 12h:12h dark light cycle. The mice were weighed once a week in order to ensure they were healthy and there was no unprecedented weight loss. Following experimental period, the mice were perfused using Tyrodes buffer containing heparin and the tissues were fixed with 10% formalin.

Water regimen

Transgenic mice were further divided into two groups with five mice in each group. The untreated group received regular drinking water whereas the treatment group received Ubisol-Q_{10} supplemented drinking water at a concentration of 200 µg/ml which means 50 µg/ml of the CoQ_{10}. Ubisol-Q_{10} was purchased from Zymes LLC (Hasbrouck, NJ) and fresh drinking water was provided to the mice once a week. Treatment started one month after their arrival and continued for a period of 18 months.

Enzyme linked immunosorbent assay

In order to measure the levels of $A\beta_{1-40}$ in the blood, ELISA was performed on the serum samples using the KHB3482 kit. Standards and sample dilutions were prepared, mixed with detection antibodies and loaded in triplicates to an antibody pre-coated ELISA plate. After incubation, the plates were washed to remove any excess unbound antigen or detection antibody

and then the HRP-conjugated antibody was added, followed by a substrate that gave a colorimetric product which could be read after stopping the reaction with sulfuric acid. Standard curve was plotted and the sample concentration was calculated from the line equation obtained from the standard curve.

Antibodies

The following antibodies were used at the mentioned dilutions to perform immunohistochemistry and assess the morphological changes and levels of various proteins in the brain. Mouse monoclonal anti- human amyloid-beta antibody (1:500; Novus Biologicals Cat NBP2-13075), mouse monoclonal anti-neuronal nuclei antibody (1:600; EMD Millipore Cat MAB 377X), rabbit polyclonal anti-glial fibrillary acidic protein antibody (1:500; Novus Biologicals Cat NB300-141) rabbit polyclonal anti-Iba-1 antibody (1:300; Cat NB100-1028), mouse monoclonal anti- proliferating cellular nuclear antigen antibody (1:400; DAKO Cat M087901-2).

Immunohistochemistry

Following perfusion the brain was extracted and stored in 10% formalin at 4 ˚C. Three days prior to sectioning using a microtome the brain was transferred to 30% (w/v) sucrose. The sections obtained were 30 µm and the entire hippocampus region was collected. On the first day of immunohistochemistry procedure, the slides were washed twice in Tris buffered saline (TBS) for 5 minutes each following which they were incubated in 1% H_2O_2 for 5 minutes in order to block any endogenous peroxidase activity. The slides were then rinsed twice for 5 minutes in TBS to wash off any excess H_2O_2 and then blocked for 30 minutes each in DAKO universal blocking solution (purchased from Diagnostics Canada Inc., Mississauga) and normal serum (followed instructions on Vecstatin ABC Kit, Vector laboratories) in order to block the binding of non-specific IgG. Following blocking the sections were incubated overnight in the primary antibody

at 4 ˚C. On the second day the slides were washed twice in TBS for 5 minutes and this time to remove any excess antibody before incubating them for 1.5 hours in the corresponding biotinylated secondary antibody present in the Vectastin ABC kit. The TBS washes were repeated and the sections were incubated in avidin biotin complex for a period of 45 minutes. The TBS washes were then carried out once again and a peroxidase substrate 3, 3ˈdiaminobenzidine was prepared and added in order to stain the specific antigenic sites on the section to which the antibody was bound to. The slides were dehydrated with first 95% ethanol and xylene and coverslipped using permount in order to visualise it under the microscope.

Congo red

The day prior to conducting the Congo red staining, 0.2% Congo red solution was prepared in saturated Nacl solution by allowing it to stir overnight. Before use, the Congo red solution was vacuum filtered and 1% v/v of 1M NaOH was added in order to make the solution alkaline. The slides subjected to Congo red staining were first rehydrated by immersing them in distilled water for 30 seconds, incubated in alkaline saturated Nacl for 20 minutes, following which they were incubated in the Congo red solution for 30 minutes. The slides were then rinsed by dipping them eight times, first in 95% ethanol, followed by 100% ethanol (repeated twice), and then incubated thrice in xylene for 5 minutes, after which they were coverslipped using permount.

Data analysis

A Leica microscope and 4.5AF software were used to visualise and capture images of the region of interest which was kept consistent across the groups. Quantification was done separately by two different experimenters. The thickness of the stratum pyramidale (CA1) region was measured using Image J at three different locations evenly distributed and then averaged.

Statistical significance of the data was calculated using SPSS and one way ANOVA was conducted. Effects from statistical analyses were considered significant at $p \leq .05$

Behavioural testing

Apparatus and materials: Two Y-mazes, constructed from Plexiglas material, consisting of equidistant arms 120° apart, each with dimensions of 22 cm (length) by 6 cm (width) by 8 cm (height) were used. One Y maze was transparent and the other was translucent. Either maze could be placed in a 50 cm square grey chamber with 40 cm grey walls. The interior of the chamber was illuminated by an incandescent 60-W lamp positioned 1.5 m above the apparatus (Figure 12). A Sony Digital Video camera was also positioned near this lamp to record the mouse's movements in the Y-maze. Noldus Ethovision XT10 software was used to track the mouse's movement in the Y-maze and to determine the amount of time it explored objects during the second phase of the experiment.

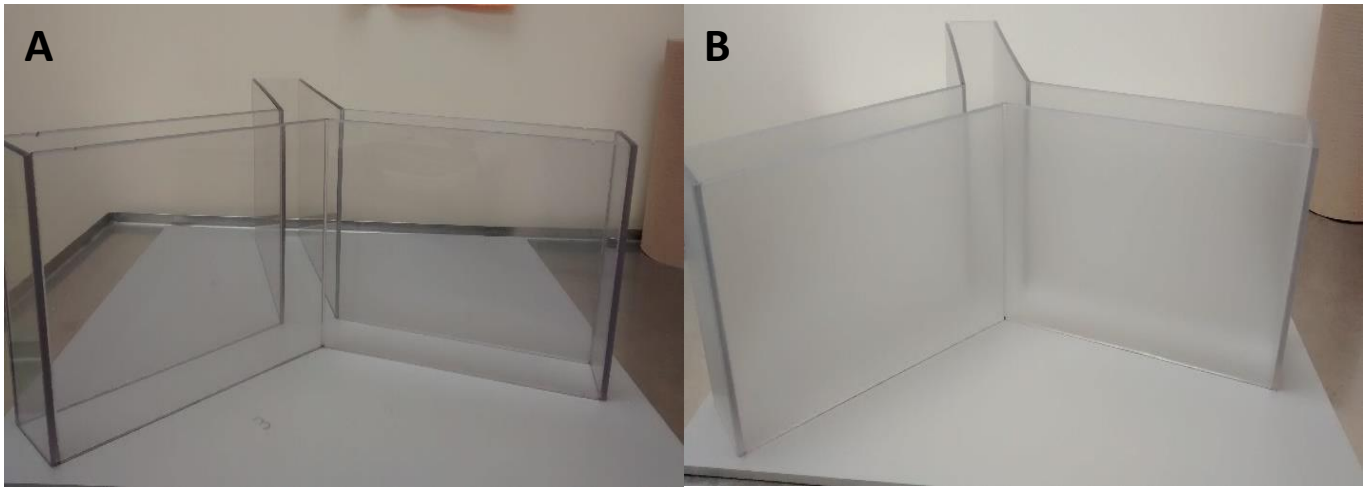


Figure 12 (A) Picture of the transparent Y-maze that was used. (B) Picture of the translucent Y-maze that was used.

Long-term reference memory task. We measured mice's habituation and spontaneous recovery of their exploration in the Y-maze. In this study, we define habituation as a decrease in overall activity or exploratory behaviour when exposed to the same, initially unfamiliar environment over repeated exposures. Of the various types of activities of the mouse in the Y-maze that the Noldus program could monitor, we report only two types of exploration in any 5-min session: the number of times a mice entered the arms and the proportion of time spent in the arms, because only these two types of behaviour showed any consistent effects as a function of treatment. A mouse was considered to have entered an arm when its body's center-point was 3 cm inside an arm. We note that mice seldom if ever backed out of an arm at this point but ran at least half way down it and often to its end before turning around and exiting the arm. This phase of the experiment consisted of two blocks of five sessions each. A daily session consisted of placing a mouse in the center of the Y-maze and allowing it to explore it for 5 min. Inter-trial intervals within each block were kept at 24 h and the interval between blocks (the 'rest' period) was 16 days. Mice were randomly assigned to begin the test in either the transparent or a translucent Y-maze which alternated over all 10 sessions. We varied exposure to these two types of mazes to determine if exploration would be affected by an animal's ability to perceive the larger chamber and if this effect was a function of group treatment. As our subsequent analysis failed to show any effects from this factor, we report animals' exploration collapsed over it. We expected that mice would habituate to the maze (i.e., reduce their exploration) over the first five trials. Their long-term memory of the maze over the 16-day rest period would be evident in the amount of spontaneous recovery of exploration when reintroduced to it. If such long-term memories were better preserved in wild-type and Ubisol-Q_{10} treated transgenic mice than in the untreated transgenic mice, the former should show less spontaneous recovery than the latter. With any

daily session, the maze was sprayed with Accel Prevention Cleaner and Disinfectant (concentration 1:40) and wiped between mice during each session.

Working Memory. We adapted the novel location / novel object recognition (NL/NOR) test [24] for the Y-maze. In this phase mice only ran in the translucent maze. The maze was modified to contain removable arm floors, two of which were made of stainless steel wire mesh and one of smooth polystyrene material. These floor cues provided additional spatial, location cues in the maze. A different object on a covered metal base, was placed at the end of two of these arms. These two objects were randomly selected from among three objects, a black large paper clasp, a round screw, and a butterfly screw, for the first familiarization trial for each mouse (Figure 13). The third object was reserved as the novel item used to replace one of the initial objects in the third trial of this task. Which of the two arms contained an object during the first, familiarization trial in this task was also randomly determined for each mouse. Each animal received three 5-min trials in the Y-maze: the first being the location familiarization trial with two objects, the second being the novel location test trial where one of these two objects had been moved to the previously 'empty' arm, and the third being the novel object test trial where the novel object had replaced the remaining unmoved object of the previous two trials. Figure 14 shows an example of these trials. Each trial was separated by a 2-min interval during which time the experimenter prepared the maze for the next trial by replacing the two objects from the first trial with identical replicates and moving one of them to the empty arm for the second trial and then for the third trial, replacing the unmoved object from the second trial with the novel object and replacing the previously moved object with an identical replicate. The maze and objects were also cleaned between trials and mice as in the first phase. These procedures were used to control for any possible differential experimenter- or subject-induced odors left on objects from a previous trial.

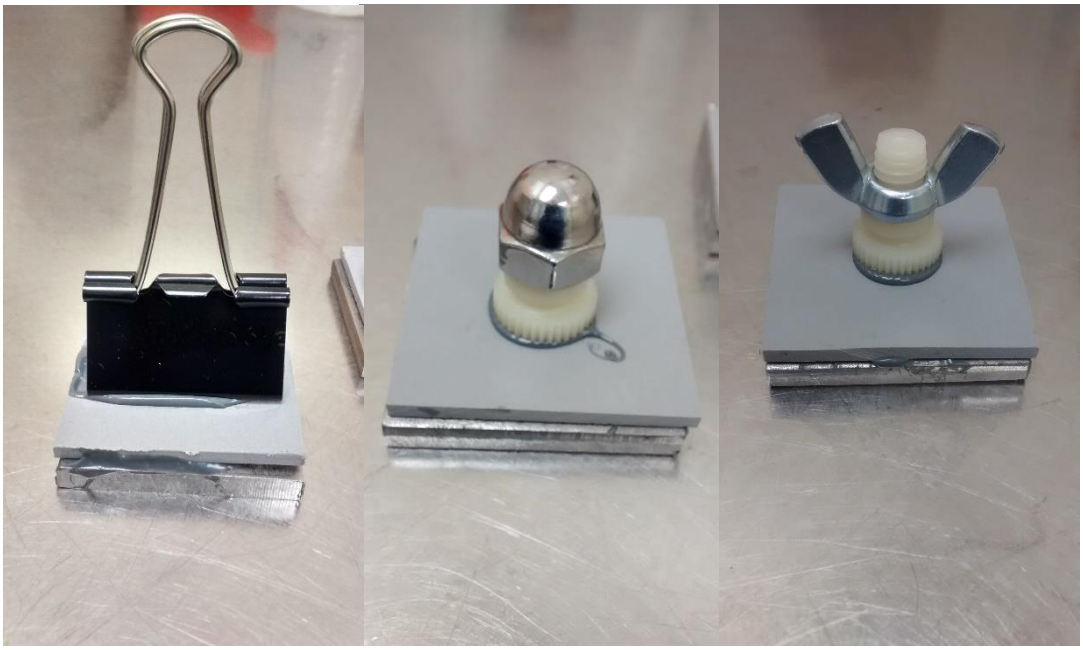


Figure 13 (A) Picture of the paper clip that was used in the NL/NOR task. (B) Picture of the round screw that was used in the NL/NOR task. (C) Picture of the butterfly screw that was used in the NL/NOR task.

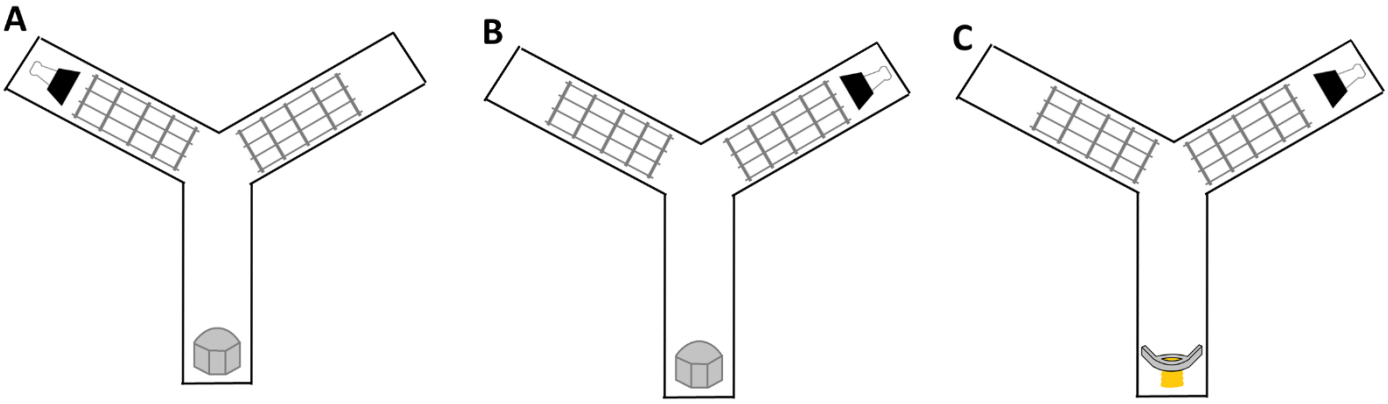


Figure 14 A schematic representation of the 3 trial NL/NOR test. (A) Familiarization trial. Two stainless steel wire mesh floors and smooth floors were placed in the arms to provide salient directional cues. Two distinct objects (paper clip and round screw shown here) were randomly placed at the ends of 2 of the arms. (B) Novel location trial. One of the objects was moved to the previously empty arm while the other object was unchanged. (C) Novel object trial. The previously unchanged object was replaced with a novel object (butterfly screw shown here) while the moved object stayed the same as in the previous trial.

In tracking a mouse's movement in the maze, the Noldus software was programmed to determine the amount of time the animal remained within 3 cm from each object during the trial as a measure of its inspection (exploration) of it. From these data we calculated the proportion of time a mouse spent near each object from its proportion of time it spent exploring both objects rather than from its time (5-min) in the maze. We used this conditional proportion measure as a more precise indicant of a mouse's object preferences as a function of its spatial and non-spatial changes because mice from each treatment condition, spent a similar overall small proportion of time inspecting both objects on any trial.

Statistical analyses

For the long-term memory task (phase 1), proportion of time spent in the arms and number of arm entries was initially analyzed by a 3 (treatment groups) by 2 (blocks) by 5 (sessions) ANOVA with repeated measures on the last two factors. As already mentioned, we collapsed these data over the within-subjects factor (maze type). To determine spontaneous recovery on the first session of block 2 from the last session of block 1 for these measures, we conducted another 3 (treatment groups) by 2 (sessions) ANOVA. We conducted subsequent analyses for simple effects based on outcomes from these statistical tests and observations as will be described in the following session of this report.

For the NL/NOR task, we conducted a 3 (treatment groups) by 2 (object locations) ANOVA with repeated measures on the second factor to determine the effects of moving an object for the second trial. To determine the effects of replacing the remaining unmoved object with a novel object for the third trial, we conducted a univariate treatment groups ANOVA. Effects from all statistical analyses were considered significant at $p \leq .05$ (one-tailed).

4.4 Results:

These transgenic mice exhibit only one of the key hallmarks of AD. Behaviour studies were performed throughout the housing period in order to assess the extent of memory deficits in a Y-maze experiment and to see if treatment with Ubisol-Q_{10} can prevent the behaviour deficits if any. The proportion of time spent exploring the arms of the Y-maze by the transgenic mice that received regular drinking water was significantly higher than the transgenic mice that received Ubisol-Q_{10} supplemented drinking water and the wild type counterpart. Separate statistical analyses within each group, revealed that only the untreated transgenic mice significantly increased their exploration, $F_{1, 5} = 8.33$, $p = .035$. These results suggest that long-term memory, as measured by the degree of spontaneous recovery or dishabituation, was better preserved in the treated transgenic and wild-type than in the untreated transgenic mice (Figure 15).

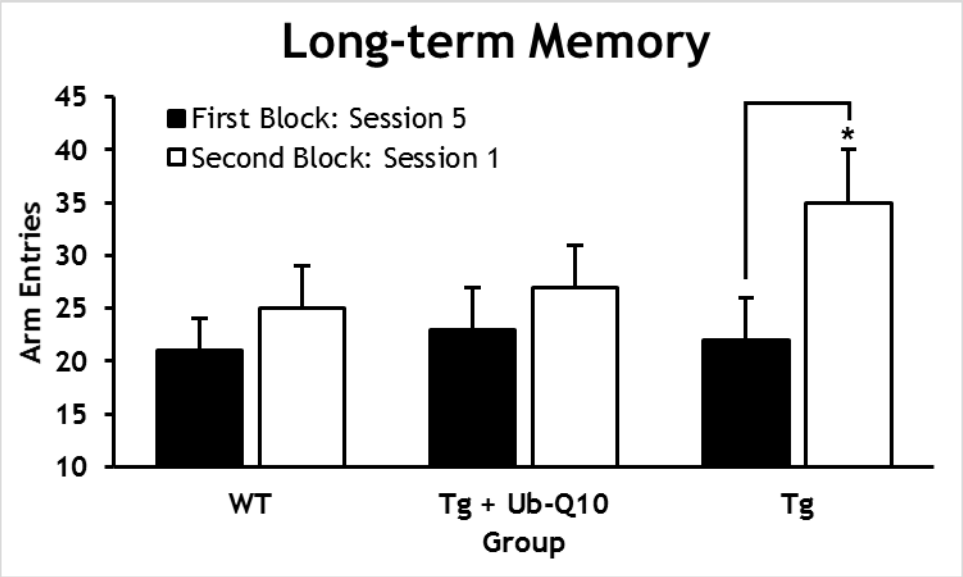


Figure 15 The number of arm entries made on the last session of the first block and the first session of the second block. Only the untreated transgenic group show a significant increase in exploration between sessions, demonstrating a large degree of spontaneous recovery or dishabituation that was not observed in the wild type or treated transgenic groups. This suggests long-term memory was preserved in the treated transgenic group compared to the untreated transgenic group. Data are represented as the Mean ± SEM. *$p < 0.05$

Working memory tests. As seen in Figure 16 and supported by a significant trial by treatment group interaction $F_{2, 16} = 3.67$, moving a familiar object from trial 1 to a new location in trial 2 promoted greater exploration of it in the wild-type and treated transgenic mice but not in the untreated transgenic mice. Further analysis of changes of exploration within each group (one-tailed *t*-tests), revealed that the treated transgenic group significantly increased its exploration, $t_{1, 6} = 2.46$, $p = .025$, the wild type showed a non-significant trend in that direction, $t_{1, 6} = 1.73$, $p = .065$, but the untreated transgenic mice showed a slight, non-significant change in the opposite direction, $t_{1, 6} = -1.58$, $p = .095$ (Figure 17). Assessment novel object recognition performance in the third trial failed to discover any main effect for treatment condition, $F_{2, 16} = 0.58$, $p < 0.57$. Further analysis of object preferences within each group revealed that only the wild-type group spent a significantly greater proportion of its total exploration time near the novel object ($.69 \pm .09$), $t_{1, 6} = 2.14$, $p = .038$, while the treated transgenic group spent only slightly more time near it ($.56 \pm .08$), $t_{1, 6} = 0.60$, $p = .285$. These results suggest that spatial working memory as measured by novel location preferences was better retained in treated transgenic and in wild-type mice than in untreated transgenic mice. Failure to replicate these effects for non-spatial working memory as measured by novel object preferences suggests that unprotected neurodegeneration in transgenic mice disrupts their spatial more than their non-spatial working memory.

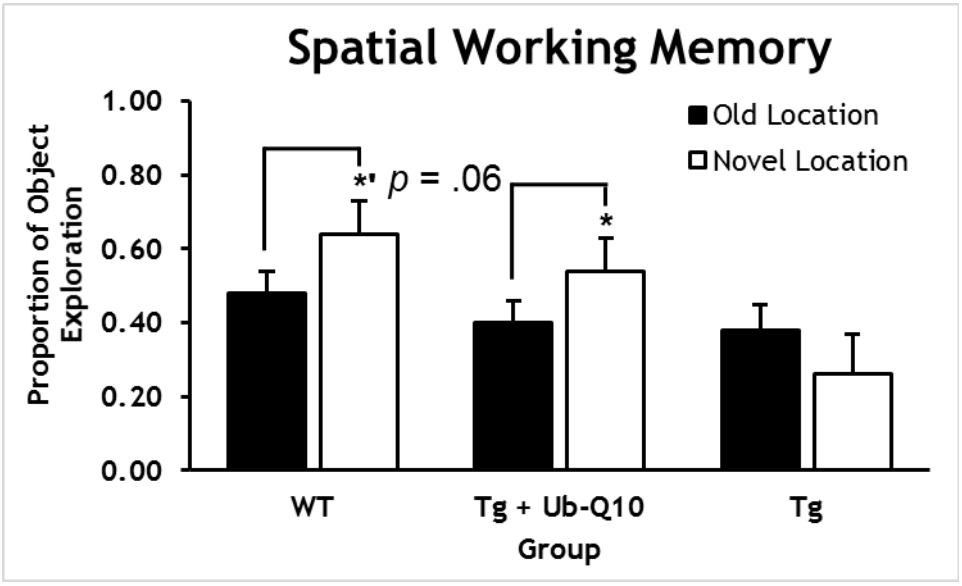


Figure 16 The proportion of time spent exploring a familiar object before and after it moved to a new location. The wild type and treated transgenic groups spend more time exploring the object in a novel location whereas the untreated transgenic group shows no difference. This suggests that spatial working memory was preserved in the treated transgenic group compared to the untreated transgenic group. Data are represented as the Mean ± SEM. *$p < 0.05$

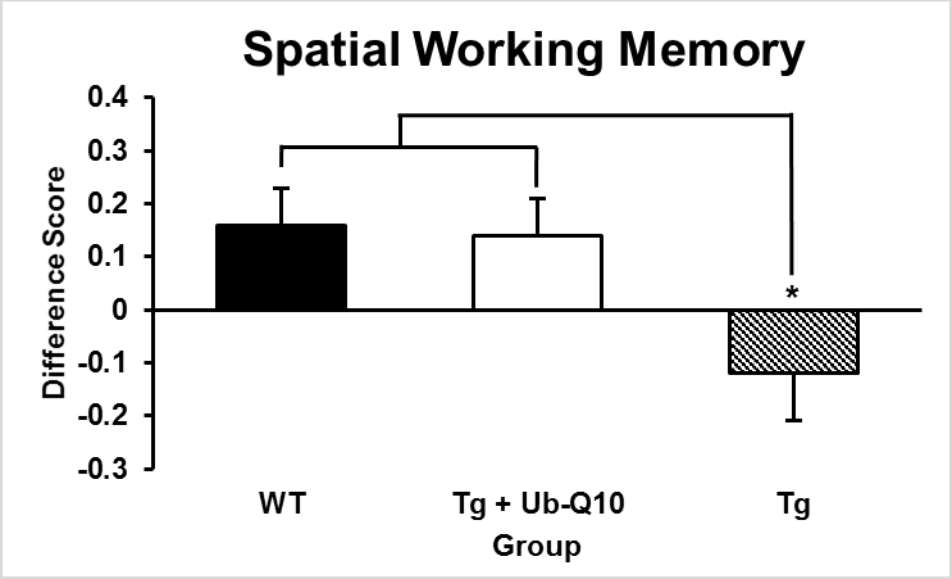


Figure 6 Novel location difference scores (exploration of the object after – before it was moved). The wild type and treated transgenic groups have a significantly greater difference score than the untreated transgenic group. This suggests that spatial working memory is preserved in the treated transgenic group compared to the untreated transgenic group. Data are represented as the Mean ± SEM. *$p < 0.05$

Following the behaviour analysis, experiments were conducted in order to see if treatment with Ubisol – Q_{10} resulted in changes in the pathological features of AD associated with the AβPP/PS1 transgenic mouse model that was used. First, the serum levels of human $A\beta_{1-40}$ was measured using ELISA in order to see if there were any observable differences across the wild type and transgenic groups. Wild type which is devoid of the human AβPP gene had no $A\beta_{1-40}$ as expected. There were however higher levels of $A\beta_{1-40}$ in the serum obtained from transgenic untreated group in comparison to the transgenic treatment group (Figure 18).

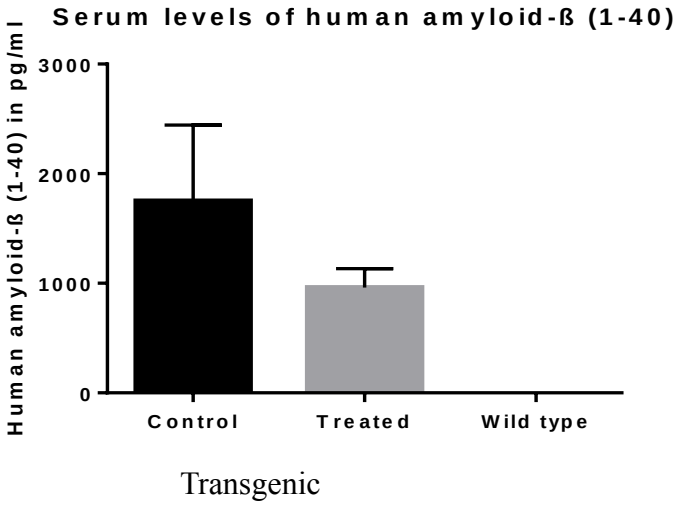


Figure 18 Serum levels of human $A\beta_{1-40.}$ A sandwich ELISA was conducted to analyze serum levels of $A\beta_{1-40}$ across the groups. The absorbance was measured at 450nm and the experiment was conducted once with serum obtained from each animal and in triplicates. The untreated transgenic group show higher levels of $A\beta_{1-40}$ in the serum whereas there is a reduction in $A\beta_{1-40}$ for the treated transgenic group. WT group shows no human $A\beta_{1-40}$ expression.

Following 18 months of treatment with either Ubisol-Q_{10} supplemented drinking water or regular water, the transgenic and wild type mice were sacrificed and the brains were subjected to immunohistochemistry with anti- human Aβ antibody or Congo red staining in order to assess the extent of amyloid plaque burden across the treatment groups. The wild type group as expected did not show immunoreactivity with human Aβ immunostaining. The transgenic untreated group had very high levels of human amyloid plaque deposits in the hippocampus region, a characteristic feature of the AβPP/PS1 transgenic mice model. In the treatment group however, there was a drastic contrast, with three mice showing near complete removal of the human amyloid plaque, and in the remaining two mice, a substantial decline in the levels of plaque was observed (Figure 19). A similar trend was observed with Congo red staining as well, further validating the immunohistochemistry results. This indicates that prophylactic treatment with Ubisol-Q_{10} is able to decrease the extent of amyloid plaque deposit in these transgenic mice model (Figure 20).

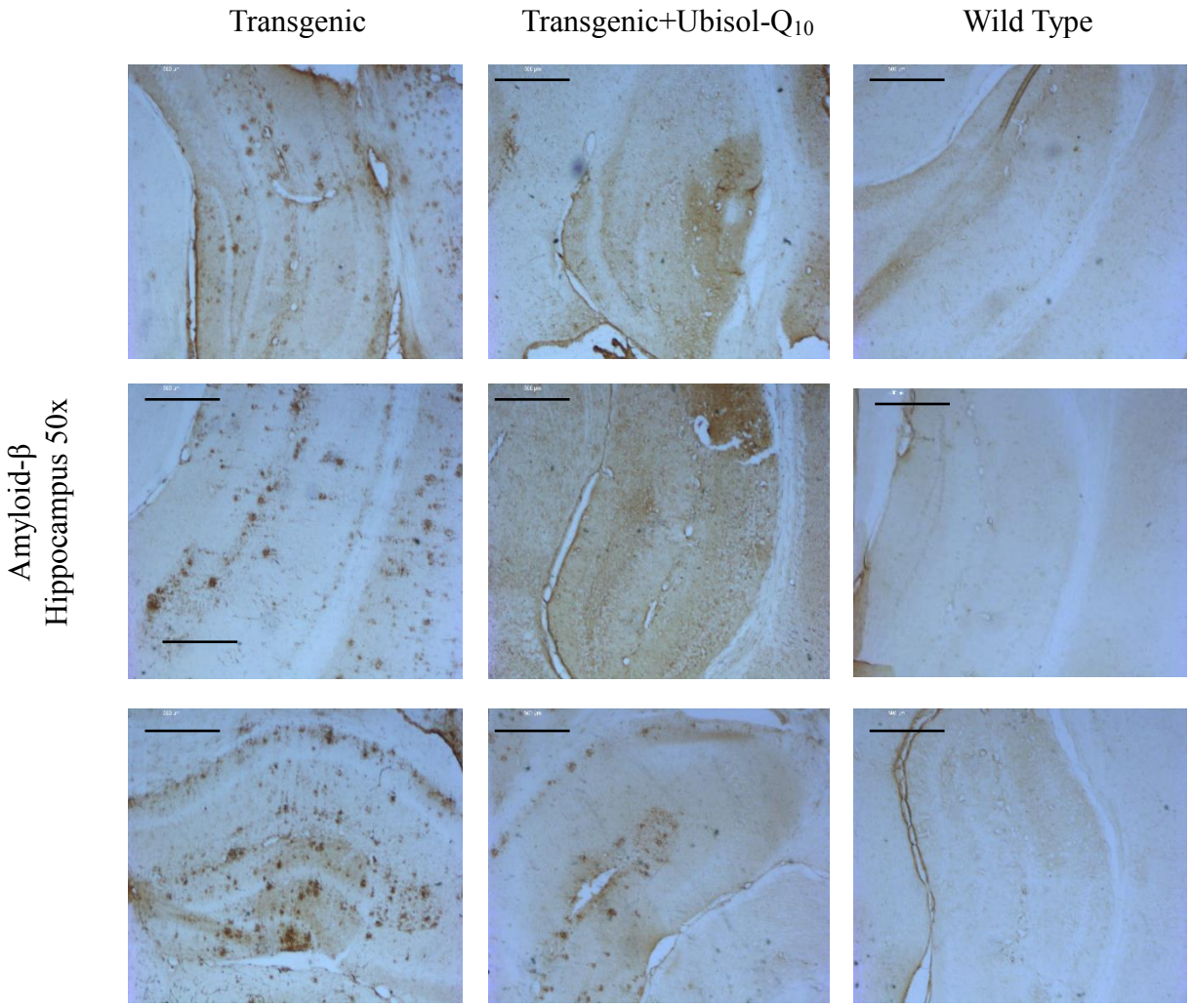


Figure 19 Staining for Aβ shows a decrease in plaque formation in Ubisol-Q_{10} treated transgenic group. Each image represents a brain section from an individual mouse. The untreated group exhibits abundant plaque formation. The first two images in the treated group demonstrate a near complete reduction of the human Aβ deposition while the third image demonstrates a mouse brain showing a drastic decrease in plaque deposition. The WT groups had no Aβ deposition. Scale bar = 500μm.

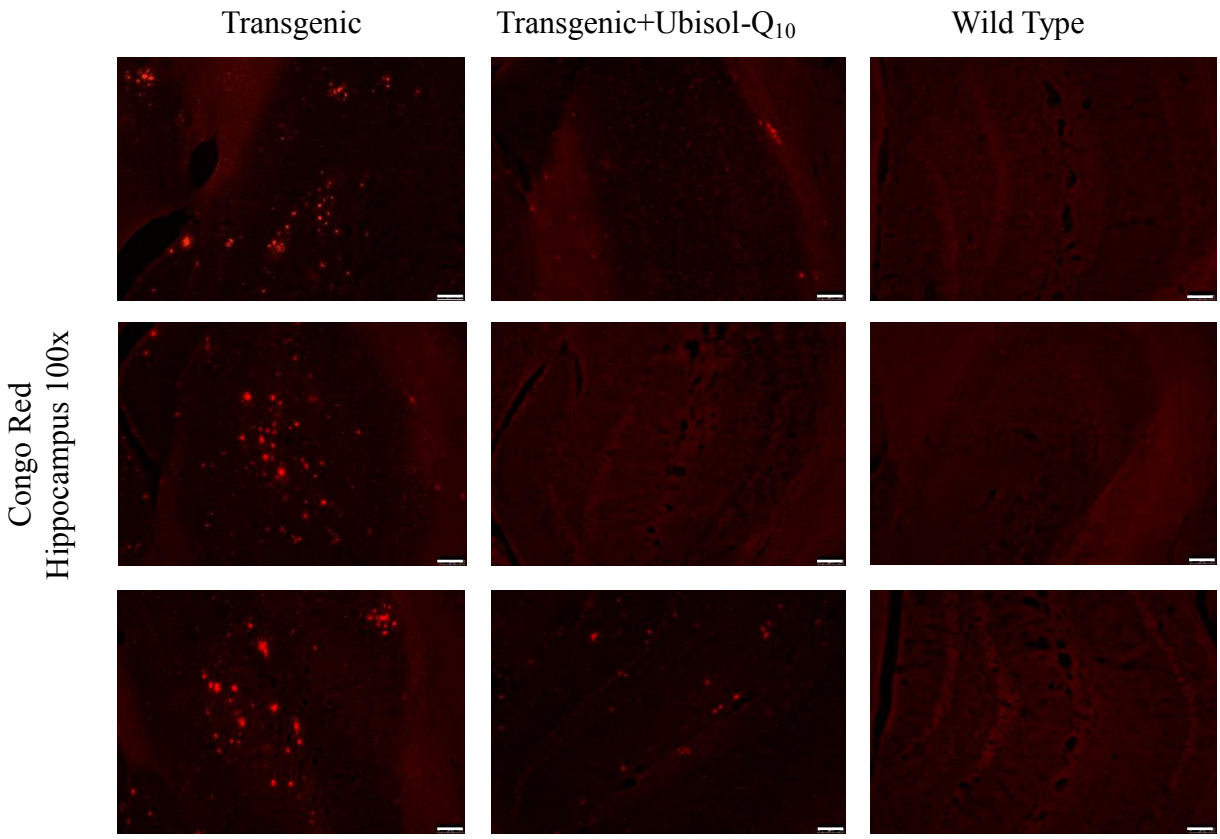


Figure 20 Congo Red staining demonstrates a decrease in Aβ plaque formation in Ubisol-Q_{10} treated transgenic group. Each image represents an individual mouse. Congo red staining was used to confirm anti-human Aβ antibody staining results. The same trend was observed where the untreated groups have higher levels of plaque deposits while the Ubisol-Q_{10} treated transgenic mice demonstrate a near-complete or reduction in plaques whereas WT has no Aβ plaque deposits. Scale bar = 100μm.

The positive results with Aβ staining lead to studying of the interacting partners of neurons, the glial cells. The morphological changes of the glial cells, namely astrocytes and microglia, were then analyzed and compared between the treatment groups. The images demonstrate increased astrogliosis in the transgenic treatment mice brain sections stained with anti-glial fibrillary antibody antibody (a marker for astrocytes, which exhibit longer and increased branching per cell). However, in comparison, the transgenic untreated and wild type groups show moderate levels of astrocyte activation with shorter and fewer branches per cell (Figure 21).

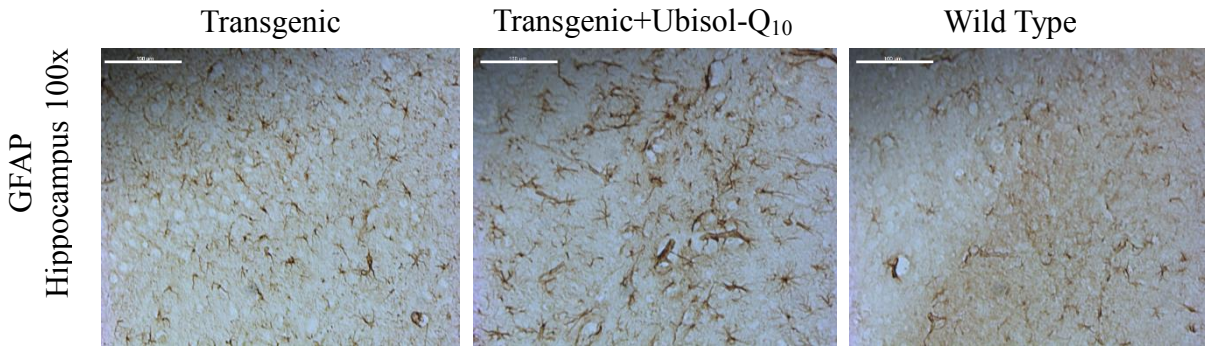


Figure 21 GFAP staining to investigate astrocyte activation. Ubisol-Q_{10} treated transgenic group demonstrate increased astrocyte activation; this is indicated by a change in morphology where there are more branches, of longer lengths per astrocyte. In comparison, the untreated and WT groups demonstrate moderate activation of astrocytes, with shorter and less branches per astrocyte. Scale bar =100μm.

The extent of microglia activation was analyzed in slides subjected to immunohistochemistry with anti-Iba1 antibody, a marker for microglia. The transgenic treatment and wild type groups have resting microglia indicated by small cell body with equally distributed, thin, long, highly branched cellular processes, whereas the transgenic untreated group have slightly reactive microglia, with large pleomorphic cell bodies that appear to clump, possibly around the amyloid plaque. Hence Ubisol-Q_{10} treatment is able to bring about changes in the morphology of glial cells in this transgenic AD mouse model and potentially alter disease pathology (Figure 22).

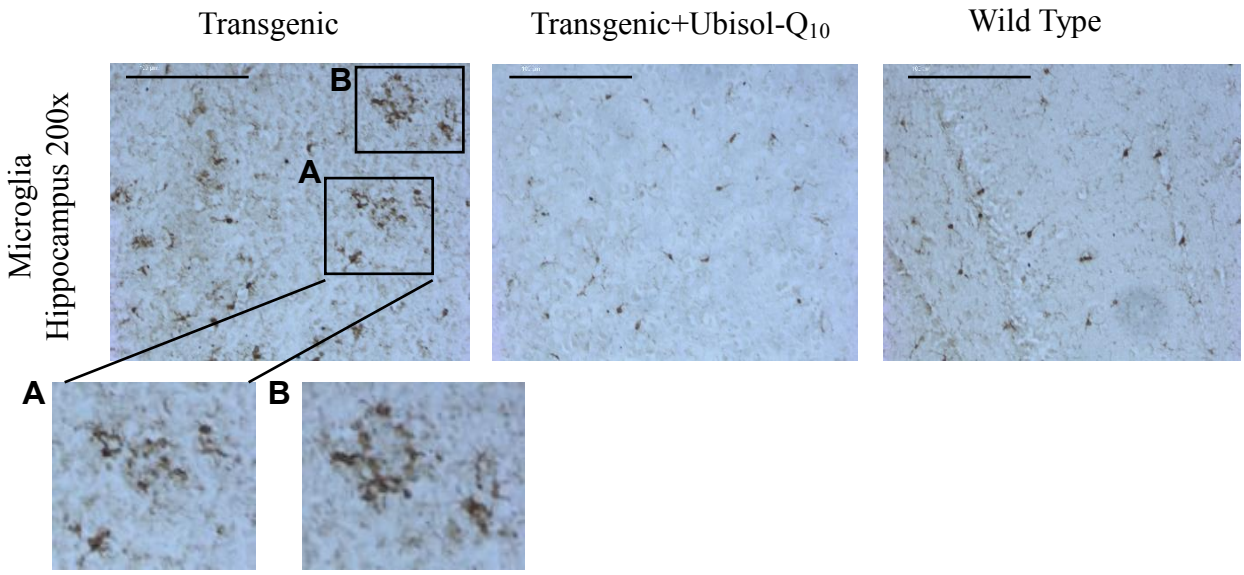


Figure 22 Iba1 staining to investigate microglial activation. The level of microglial activation is indicated by the cells morphology and pattern of gene expression. The untreated transgenic mice exhibit enhanced microglial clumping, as shown by A and B. The Ubisol-Q_{10} treated transgenic mice and WT groups both express morphology that resembles resting microglia as noted by the small cell body, with evenly located thin branching and lack any visible clumping. Scale bar = 100μm.

There is significant loss of neurons in the hippocampus region in patients suffering from AD, and histological evidence indicates thinning of the CA1 as a result of neurodegeneration. The thickness of the CA1 layer was hence measured and averaged in slides subjected to immunohistochemistry using anti-NeuN antibody, a marker for differentiated neurons and as described in Cerbai et al., 2012 (Figure 23A) [25]. There were no significant differences in the thickness of the CA1 even though the transgenic untreated group had a thinner band in comparison to the transgenic treatment and wild type groups. (Figure 23B).

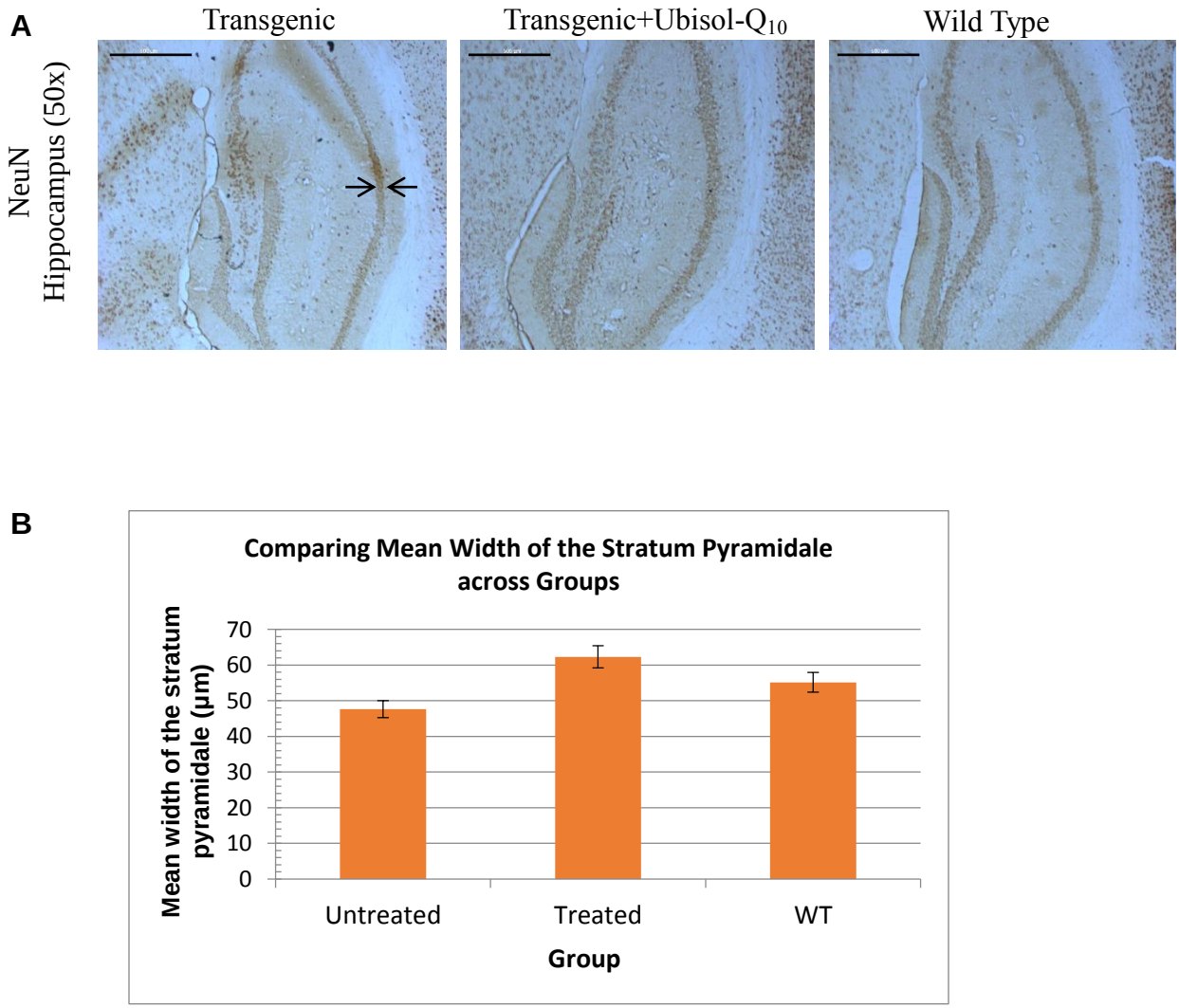


Figure 23 Quantitative analysis of the stratum pyramidale thickness. (A) Microscope images show NeuN immunohistochemistry staining at 50x magnification. The thickness of the CA1 was measured on three equally distributed locations (within a distance of 150µm) using Image J software (arrows indicate the location of the measurement taken). Measurements were averaged over three brain sections per animal. Scale bar = 500µm. **(B)** Quantification of the width of the CA1 was made using Image J software. No statistical difference was seen, but a general trend was observed where the untreated group showed a thinning of the CA1 compared to the treated group.

Next, hippocampus neurogenesis was assessed by performing immunostaining with anti-proliferating cellular nuclear antigen (PCNA) antibody. There was a higher degree of immunoreactivity indicating neurogenesis in the transgenic treatment group and no immunoreactivity indicating no neurogenesis observed in the transgenic untreated and wild type groups (Figure 24).

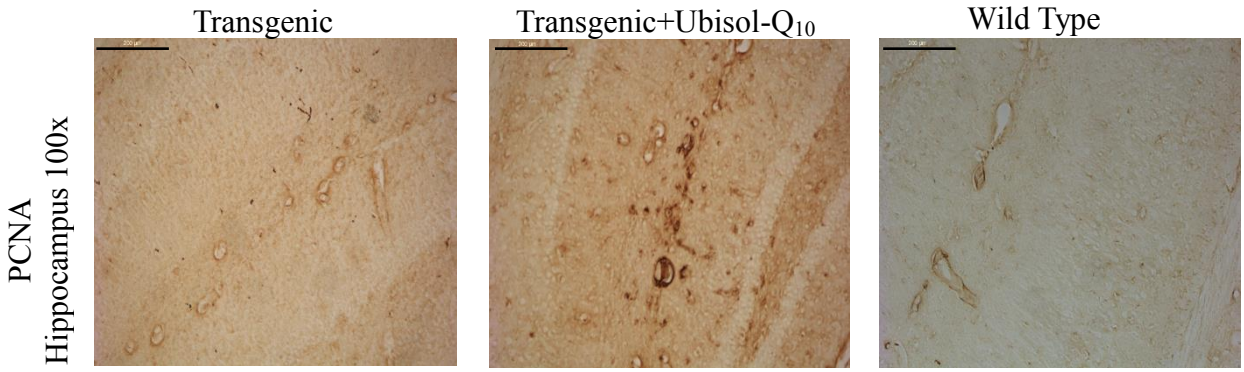


Figure 24 Results using PCNA immunohistochemistry staining. Microscope images of the hippocampus at 100x magnification indicate increased PCNA in the Ubisol-Q_{10} treated transgenic mice whereas both the untreated and WT groups show reduced levels or no PCNA immunoreactivity. Scale bar = 200μm.

4.5 Discussion:

Previous research has shown that hippocampal damage, either by directly lesioning the hippocampus [26,27,28,29,30] or by indirectly affecting the hippocampus through the development of AD-related transgenic models [31,32], primarily affects long-term memory and spatial working memory. Based on these findings, we conducted behavioural tests of long-term memory and spatial working memory to assess the potential therapeutic effects of Ubisol-Q_{10}. To examine long-term memory, we determined the degree of dishabituation in a Y-maze; that is, the extent to which mice spontaneously recover a previously habituated exploratory response following a long rest from exposure to the Y-maze. The assumption being that the amount of spontaneous recovery measures some degree of forgetting about prior experience in the maze. This hypothesis is based on the notion that habituation of an animal's reaction to a novel event and its spontaneous recovery following a longer period (rest) away from the event reflects a basic learning and memory process. We saw that the untreated transgenic mice showed greater spontaneous recovery compared to treated transgenic mice and wild type mice. The long-term memory was thus preserved in the transgenic mice that received Ubisol-Q_{10} treatment. We also conducted a novel location / novel object recognition (NL/NOR) task in the Y-maze, similar to that originally used by Benice et al., 2006 [33]. In their study, mice were placed in an open field with three distinct objects each located in a different corner. The three objects remained in those locations over the first three trials, followed by a fourth "novel location" trial where one of the objects was moved to the fourth corner, and finally by a fifth "novel object" trial where one of the previously unmoved objects was replaced by a novel object. According to Benice, increased exploration of the moved object suggests the mouse had remembered the objects' spatial features, providing insight into their spatial working memory. Additionally, increased

exploration of the novel object suggests the mouse remembered the previous objects' non-spatial features allowing it to recognize which of the objects had been replaced by a new one. Taken together, these measures provide a robust indication of spatial and non-spatial working memory processes. Prophylactic treatment with Ubisol-Q_{10} was able to prevent the deficits in the spatial-working memory in these double transgenic AD mice in comparison to the untreated group which showed less exploration of the moved object.

Mitochondrial dysfunction and oxidative stress play a crucial role in the pathophysiology of AD and has been shown to precede the key pathological changes associated with the disease. Hence, development of antioxidant drugs that can help improve mitochondrial health holds promise as effective therapies for AD. We have previously shown that Ubisol-Q_{10} is able to reduce the levels of ROS, ameliorate mitochondrial dysfunction, and delay premature senescence in PS1 mutated AD familial type 3 fibroblasts [22].

In this report we have demonstrated the efficacy of prophylactic administration of Ubisol-Q_{10} in altering behavioral deficits and pathology associated with the AβPP/PS1 transgenic AD mouse model.

Administration of Ubisol-Q_{10} for a period of 18 months is also able to decrease the load of circulating human $A\beta_{1\text{-}40}$ as well as remove human amyloid plaque in these transgenic mice genetically predisposed to amyloid plaque deposition at 6-8 months of age. The differences in the plaque deposition in the transgenic treatment group could be attributed to Ubisol-Q_{10} being provided in the drinking water in their group cages and differences in water intake.

Previous studies in our lab has shown that Ubisol-Q_{10} is not toxic and is able to cross the blood brain barrier, reach the target organ in order to provide significant neuroprotection and halt neurodegeneration in rodent models of PD [20]. The current findings suggest that Ubisol-Q_{10}

could be a novel treatment option for AD and could ameliorate the pathological features related to mitochondrial dysfunction, oxidative stress, and the accumulation of protein aggregation and autophagy.

There could be multiple factors involved in the pathophysiology of AD as there is a wide array of clinical manifestations observed in patients suffering from AD. Currently there is no rodent model available that can fully mimic the decline in cognitive capacities as well as all the key pathological features observed in AD. However, we were able to show that Ubisol-Q_{10} was able to decrease the Aβ deposition in the AβPP/PS1 transgenic mice model. Studies suggest that when there is an imbalance between accumulation and clearance of Aβ, it leads to an increase in the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ which has the tendency to aggregate and form plaques [34]. There are two different schools of thought which suggests that this plaque deposition could either precede or follow mitochondrial dysfunction and oxidative stress. These results suggest that Ubisol-Q_{10} is able to prevent the Aβ deposition by bringing about mitochondrial stability, improving the efficiency of energy production, and decreasing the levels of ROS, which also confirms the role played by mitochondria in ageing and in AD.

Neurodegeneration in the hippocampus, one of the key pathological features of AD is not observed in the AβPP/PS1 transgenic mice, and we did not observe changes in the thickness of the CA1 band. There was no observable change in the neuron density in the vicinity of plaque as well. In order to understand the effect of Ubisol-Q_{10} in preventing hippocampal neurodegeneration, studies should be carried out in AβPP/PS1K1 transgenic mice which show significant loss of CA1 pyramidale cells.

Hippocampus neurogenesis has physiological significance and it has been shown that blocking of neurogenesis leads to impairment of hippocampus related-learning [35]. The transgenic

treatment group showed increased neurogenesis when stained with anti-PCNA antibody, a marker for highly proliferating cells in G1 and S phase. Hence, hippocampal neurogenesis observed with Ubisol-Q_{10} treatment could be attributed to preserving long-term memory in the transgenic group. However, immunohistochemistry with a neuronal stem cell marker such as Nestin will provide a better understanding of the neurogenesis observed in the treatment group.

Interaction between neurons and glial cells is important for maintaining normal brain homeostasis and hence it is essential to elucidate their contribution and role in disease pathophysiology. PET studies suggest that the astrocytes are highly activated in the initial stages of AD following which there is a decline [36]. The astrocytes could initially aid in slowing down the disease progression following which it is unable to do so due to the toxic environment created by Aβ and ROS. Astrocytes activation observed in the transgenic treatment group along with reduction in Aβ plaque and increased neurogenesis shows that the neuroprotective property of astrocytes is resumed with Ubisol-Q_{10}. The mechanism of astrocytes activation is however still unclear, but they could play a role in restoring neurogenesis in the transgenic treatment group by providing the essential growth factors.

In contrast to astrocytes there are resting microglia in the transgenic treatment group in comparison to transgenic untreated group which have more reactive microglia that clump around the Aβ plaque. The presence of reactive microglia, followed by increase in pro-inflammatory cytokines and neuroinflammation is a viscous cycle and an attempt to restore normal physiological conditions [37]. The presence of resting microglia in the transgenic treatment group similar to the wild type group suggests that Ubisol-Q_{10} is able to reduce the toxic effects posed by the Aβ in these transgenic mice model.

Even though the mechanism of neuroprotection by Ubisol-Q_{10} needs to be further elucidated, these results suggest the significant role of mitochondria and how reducing ROS to the basal levels of is effective in restoring physiological conditions and reversing the threats posed by mitochondrial dysfunction, one of the key hallmarks of ageing.

Authors' contributions

Krithika Muthukumaran, Alexandra Marginean, Austin Elliott, Annie Kanwar, Nicholas Guilbeault, Jerome Cohen and Siyaram Pandey contributed to the planning and execution of the experiments and writing the manuscript. Krithika Muthukumaran, Alexandra Marginean, Austin Elliott, Annie Kanwar were involved in weighing the mice, animal care, feeding of different water regiments, dissections, immunohistochemical analysis and biochemical analysis. Jerome Cohen, and Nicholas Guilbeault were involved in the design and execution of behavioural tests. Marianna Sikorska prepared water-soluble CoQ_{10} and placebo formulations. All authors read and approved the final manuscript.

4.6 References:

1. McKhanna GM, Knopman DS, Chertkowd H, Hyman BT, Jack CR, Kawash CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carillot MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging and the Alzheimer's Association workgroup. *Alzheimer's & Dementia* 1-7.
2. Rhein V, Eckert A (2007) Effects of Alzheimer's amyloid-beta and tau protein on mitochondrial function-role of glucose metabolism and insulin signalling. *Archives of Physiology and Biochemistry 113*(3), 131–141.
3. Murrell J, Farlow M, Ghetti B, Benson M D (1991). A Mutation in the Amyloid Precursor Protein Associated with Hereditary Alzheimer's disease. *Science* 254(5028), 97-99.
4. Sherrington et al (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375(6534), 754-60.
5. Levy-lahad AE et al (1995). Candidate Gene for the Chromosome 1 Familial Alzheimer's Disease Locus. *Science* 269(5226), 973-7.
6. Lipton SA (2004). Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx : The Journal of the American Society for Experimental NeuroTherapeutics* 1(1), 101–10.
7. Hughes RE, NikolicK, Ramsay RR (2016) One for All? Hitting Multiple Alzheimer's Disease Targets with One Drug. *Frontiers in Neuroscience* 10, 1–10.
8. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. *Cell* 153(6), 1194-1217.

9. Dantuma NP, Bott LC (2014) The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution *Front. Mol. Neurosci.* 7, 70.
10. Lilienbaun A (2013). Relationship between the proteasomal system and autophagy. *Intl J of Biochem Mol Biol.* 4(1): 1-26.
11. Uttara B, Singh AV, Zamboni P, Mahajan RT (2009) Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology* 1, 65-74.
12. Hardy J, Allsop, D (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci*, 12, 383-388.
13. Selkoe, DJ. (2001). Alzheimer's disease: Genes, proteins, and therapry. *Physiol Rev* 81(2), 741-766.
14. Parker WD Jr, Parks J, Filley CM, Kleinschmidt-DeMasters BK. (1994) Electron transport chain defects in Alzheimer's disease brain. 44(6),1090-6.
15. Spindler M, Beal MF, Henchcliffe, C (2009) Coenzyme Q10 effects in neurodegenerative disease. *Neuropsychiatric Disease and Treatment* 5, 597-610.
16. Cleren C, Yang L, Lorenzo B, Calingasan NY, Schomer A, Sireci A, Beal MF (2008) Therapeutic effects of coenzyme Q10 (CoQ_{10}) and reduced CoQ_{10} in the MPTP model of Parkinsonism. *Journal of neurochemistry* 104(6), 1613-1621.
17. Beal et al (2014) A randomized clinical trial of high-dosage coenzyme Q10 in early Parkinson disease: no evidence of benefit. *JAMA Neurol* 71(5), 543-52.
18. Huntington Study Group. (2001). A randomized, placebo-controlled trial of coenzyme Q10 and remacemide in Huntington's disease. *Neurology* 57(3), 397-404.

19. Borowy-Borowski H, Sodja C, Docherty J, Walker PR, Sikorska M (2004) Unique Technology for Solubilization and Delivery of Highly Lipophilic Bioactive Molecules. *Journal of Drug Targeting*, 12(7), 415-424.

20. Muthukumaran K, Leahy S, Harrison K, Sikorska M, Sandhu, JK, Cohen J, Keshan C, Lopatin D, Miller H, Borowy- Borowski H, Lanthier P, Wienstock S, Pandey S (2014) Orally delivered water soluble Coenzyme Q10 (Ubisol-Q_{10}) blocks on-going neurodegeneration in rats exposed to paraquat: Potential for therapeutic application in Parkinson's disease. *BMC Neurosci* 14, 21.

21. Muthukumaran K, Smith J, Jasra H, Sikorska M, Sandhu JK, Cohen J, Lopatin D, Pandey S (2014) Genetic susceptibility model of Parkinson's disease resulting from exposure of DJ-1 deficient mice to MPTP: Evaluation of neuroprotection by Ubisol– Q_{10}. *Journal of Parkinson's disease* 4, 523-530.

22. Naderi J, Lopez, C, Pandey, S. Chronically increased oxidative stress in fibroblasts from Alzheimer's disease patients causes early senescence and renders resistance to apoptosis by oxidative stress. *Mech. Ageing Dev.* 127 (1), 25–35.

23. Ma D, Stokes K, Mahngar K, Domazet – Damjanov D, Sikorska M, Pandey S. Inhibition of stress induced premature senescence in presenilin-1 mutated cells with water soluble Coenzyme Q10. *Mitochondrion* 17, 106–115.

24. Benice TS, Raber J. (2008). Object recognition analysis in mice using nose-point digital video tracking. *Journal of Neuroscience Methods 168*(2), 422-430.

25. Cerbai, F, Lana D, Nosi D, Petkova-Kirova P, Zecchi S, Brothers HM, Wenk GL, Giovannini MG (2012). The neuron-astrocyte-microglia triad in normal brain ageing and in a model of neuroinflammation in the rat hippocampus. *PloS one* 7(9), e45250.

26. Aggleton, JP, Hunt PR., Rawlins JNP (1986) The effects of hippocampal lesions upon spatial and non-spatial tests of working memory. *Behavioural Brain Research*, 19(2), 133-146.
27. Broadbent NJ, Squire LR, Clark RE (2004) Spatial memory, recognition memory, and the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* 101(40), 14515-14520.
28. Forwood SE, Winters BD, Bussey TJ (2005) Hippocampal lesions that abolish spatial maze performance spare object recognition memory at delays of up to 48 hours. *Hippocampus* 15(3), 347-55.
29. Save E, Poucet B, Foreman N, Buhot MC (1992) Object exploration and reactions to spatial and nonspatial changes in hooded rats following damage to parietal cortex or hippocampal formation. *Behavioral Neuroscience* 106(3), 447.
30. Stupien G, Florian C, Roullet P (2003) Involvement of the hippocampal CA3-region in acquisition and in memory consolidation of spatial but not in object information in mice. *Neurobiology of Learning and Memory* 80(1), 32-41.
31. Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen, P, Wright K, Saad I, Mueller R, Morgan D, Sanders S, Zehr C, Campo K, Hardy J, Prada CM, Eckman C, Younkin S, Hsiao K, Duff K (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nature Medicine* 4(1), 97-100.
32. Billings LM, Oddo S, Green KN, McGaugh, JL, LaFerla F (2005) Intraneuronal Aβ causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* 45(5), 675-688.

33. Benice TS, Rizk A, Kohama S, Pfankuch T, Raber J (2006) Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. *Neuroscience* 137(2), 413-423.
34. Salminen A, Kaarniranta K, Kauppinen A, Ojala J, Haapasalo A, Soininen H, Hiltunen M (2013) Impaired autophagy and APP processing in Alzheimer's disease: The potential role of Beclin 1 interactome. *Progress in Neurobiology* 106, 33–54.
35. Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci* 11(5), 339-350.
36. Rodriguez-Vieitez et al., (2016). Diverging longitudinal changes in astrocytosis and amyloid PET in autosomal dominant Alzheimer's disease. Brain, 139, 922-936.
37. Heneka M T, O'Banion KM (2007) Inflammatory processes in Alzheimer's disease. *Jour Neuroimmunology* 184, 69-91.

Chapter 5

General discussion

The world is facing an unprecedented situation, where soon the number of people at extreme old age will outnumber children. But the key question is whether a longer lifespan will be accompanied with a longer period of a good quality life that includes a sense of well-being and social engagement or whether this longer lifespan is filled with illness, disability and dependence. The two most common age-dependent neurodegenerative diseases, Alzheimer's disease (AD) and Parkinson's disease (PD), have been familiar to neuroscientists for decades, however, we do not have a comprehensive understanding of the disease pathogenesis or treatment options that can successfully halt the disease progression.

For my thesis, I established the efficacy of a water soluble formulation of CoQ_{10} (Ubisol-Q_{10}), which is a well-known antioxidant, in animal models of two most common age related neurodegenerative diseases, PD and AD. For the first time, bioavailability studies were conducted with this water soluble formulation and I show that Ubisol-Q_{10} crosses the blood brain barrier (BBB) and it is able to provide significant neuroprotection at relatively low doses. In an environmental toxin paraquat (PQ) model of PD, Ubisol-Q_{10} successfully halts the progression of neurodegeneration, when provided therapeutically and in addition ameliorates the behavioural deficits as well. Also, prophylactic administration of Ubisol-Q_{10} in a transgenic MPTP DJ-1 animal model was able to provide significant neuroprotection. Furthermore, in a double transgenic (APP/PS1) AD model, which starts to develop amyloid plaque deposits at 6-8 months of age prophylactic treatment with Ubisol-Q_{10} was able to reduce the load of amyloid plaque deposits, and protect long term memory. The biochemistry results were complimented by behaviour studies conducted by our collaborator Dr. Cohen and his team.

Mitochondrial dysfunction is one of the hallmarks of ageing, and paired with oxidative stress, these two key features are known to contribute to the pathophysiology of both AD and PD. This increase in oxidative stress could be attributed to increased accumulation of pro-oxidant factors with age, decline in antioxidant defense, or a combination of both factor [1].

The discovery of toxins such as MPTP, 6-hydroxydopamine (6-ODHA) and PQ, that can exhibit specific loss of dopaminergic neurons in the SNpc region of the brain in animal models exposed to these toxins, has made it possible to decipher their mechanism(s) of toxicity. Similarly, with the identification of genes linked to familial cases of PD, such as Parkin and PINK1, it is now easier to develop a more comprehensive understanding of the factors that contribute to the pathogenesis of PD. So far, they all converge on mitochondrial dysfunction and increase in oxidative stress. Evidence in these available animal models of PD highlights the significance of mitochondrial dysfunction and increase in oxidative stress in the disease pathogenesis [2].

Similarly, in the case of AD, mitochondrial dysfunction is a hallmark that leads to changes in structure, motility and dynamics in addition to synaptic deficits, and accumulation of damaged mitochondria, all of which are commonly observed in sporadic and familial cases of AD [3].

Current treatment options available for AD and PD only provide symptomatic relief and do not prevent the disease progression. Hence one of the challenges is to develop animal models that can closely mimic the disease pathology and symptoms in order to be able to effectively test the various therapeutic options. Most of the animal models available for PD utilise an acute dose of the toxin and very few where the animals are exposed to the toxin over a period of time [2]. For the first time, I was able to show that an environmental toxin model, a PQ rat model of PD receiving a chronic dose of the toxin exhibits slow progressive neurodegeneration similar to what

is seen in patients suffering from PD. Hence the PQ rat model very closely resembles what could happen in sporadic cases of PD.

Keeping mitochondrial dysfunction and oxidative stress as the targets for treatment, one of the ways to approach treating these two diseases involves using an antioxidant such as Coenzyme Q_{10} (CoQ_{10}). It is an important part of the mitochondrial ETC and transfers electrons from complex I and II to complex III, and is an efficient free radical scavenger [4].

Previously, clinical trials were conducted with the oil soluble formulation of CoQ_{10} for PD and Huntington's disease, however, it failed due to poor availability [4,5]. When the required effective dose that was needed for significant neuroprotection in animal models was translated to a human dose, it was way above the FDA approved dose. In this thesis, we have used a water soluble formulation of CoQ_{10}, Ubisol-Q_{10}, which is bioavailable, crosses the BBB, and provides neuroprotection in the animal models at much lower doses, and hence, when translated, it would be available to humans at a low effective dose of 6 mg/kg/day as well, within the FDA approved guidelines. Furthermore, Ubisol-Q_{10}, when tested *in vitro* was able to quench ROS, bring about mitochondrial stability, and increase ATP production and hence mitochondrial bioenergetics [6,7].

Toxicokinetics studies of PQ suggest that PQ is slowly released from the cells and it tends to have a toxic effect for prolonged periods. Ubisol-Q_{10} functions by acting as a ROS scavenger to reduce the toxic effects of the ROS generated in the PQ redox cycle [8]. Bioavailability studies also show that Ubisol-Q_{10} does not get accumulated in the system and the levels of CoQ_{10} in the brain decreases over time. Therefore, our results suggest that in order to provide prolonged neuroprotective effect, Ubisol-Q_{10} treatment must be continuous as the neurodegeneration

process resumes once the treatment is withdrawn. This study has to be carried out over a longer time period in order to get a more conclusive understanding.

In order to test Ubisol-Q_{10} in an animal model that closely mimics what is observed in patients suffering from PD, a PQ environmental toxin animal model was developed, which showed slow progressive loss of dopaminergic neurons in the SNpc region of the brain. Ubisol-Q_{10} was able to halt neurodegeneration when administered following the induction of neurodegeneration in the PQ rat model. This suggests that Ubisol-Q_{10} could be administered to PD diagnosed patients, who normally exhibit a loss of 50-60% of dopaminergic neurons at the time of diagnosis, in order to slow down or halt the disease progression. A better understanding of PQ as a neurotoxin and Ubisol-Q_{10} as a therapeutic would have been obtained if the rodents were housed for a longer period and not sacrificed only at two time points, four or eight weeks following the PQ injection regiment. It would have been interesting to test multiple doses of Ubisol-Q_{10} to examine whether there are dose-dependent changes in the levels of neuroprotection offered by this formulation.

For better representation of familial cases of PD in humans, we used a DJ-1 transgenic knock out mouse model, in order to see if Ubisol-Q_{10} can be used as a preventative agent in humans who have a familial history of PD. DJ-1 is an antioxidant and the loss of DJ-1 function contributes greatly to the susceptibility of dopaminergic neurons to MPTP, as such, it was not a surprise that prophylactic treatment with the antioxidant Ubisol-Q_{10} was effective.

Given the number of studies showing the significance of oxidative stress and mitochondrial dysfunction in triggering Aβ accumulation thus to the pathophysiology of AD, Ubisol-Q_{10} was tested in an APP/PS1 transgenic mice model. Ubisol-Q_{10} was administered prophylactically in this animal model that starts to show Aβ plaque deposition at around 6-8 months of age; with treatment, Aβ plaque accumulation was reduced drastically. If Aβ deposits is a feature associated

with ageing and is caused by inadequate clearance of plaques via autophagy due to the cumulative effects of increasing oxidative stress with age, then Ubisol-Q_{10} perhaps helps restore neuronal health and mitochondrial bioenergetics, thereby resuming basal autophagy. Although there is disagreement on whether Aβ serves a pro- or anti-oxidant effect, the outcome of Ubisol-Q_{10} remains the same in possibly aiding to decrease the levels of ROS generated in an ageing brain.

This study needs to be repeated for more conclusive results, where all mice receive a consistent treatment schedule with Ubisol-Q_{10} rather than having Ubisol-Q_{10} provided in the drinking water of the group cage. Similar experiments would have to be conducted on AD animal models that display other hallmarks of AD, such as neuronal loss or NFTs. This would help understand the efficacy of Ubisol-Q_{10} in preventing the other pathological features associated with AD and the role of mitochondrial dysfunction and oxidative stress in the other abnormalities that arise in AD. There are a number of studies that show caspase dependence in the neurodegenerative diseases, such as AD and PD and prevention of cell death with the addition of caspase inhibitors further validates this [9, 10, 11]. However, the trigger for the neurodegenerative process is still unclear; perhaps the trigger lies in changes in physiological processes or in the toxic effects of misfolded proteins. This suggests that in order to be able to halt neurodegeneration, therapeutics should aim at targeting multiple events (increased ROS, mitochondrial dysfunction, release of cytochrome c, caspase activation, inability of mitochondria to act as an effective calcium buffer) and the interacting pathways. Although Ubisol-Q_{10} might not be able to address all the pathways involved in the induction of apoptosis in the neurons, it could help alleviate some of the factors that lead up to the induction of cell death pathways, by acting as an antioxidant.

Microglia plays a role in the pathogenesis of neurodegenerative diseases, especially neuroinflammation. Resting microglia have a ramified morphology and play a role in maintenance of normal brain homeostasis, synaptic plasticity, clearing of damaged neurons, brain development and establishing memory [12]. However, in the case of inflammation, microglia assume a more amoeboid-like morphology and based on the expressed proteins, can be pro-inflammatory or anti-inflammatory. Studies have shown that microglia are found around Aβ in AD and could either enable clearing up the plaques or producing an inflammatory response [13]. Hence the nature of microglial activation could determine the response microglia has to Aβ plaque. In the control transgenic mice, we observed moderately reactive microglia with increased clumping of microglia, which may have been induced by the Aβ plaque deposits. In the case of PD, based on various studies on the animal models, microglia seems to have an important role in participating in oxidative stress, and enabling the loss of dopaminergic neurons. This increased oxidative stress is thought to activate and cause pro-inflammatory responses in the activated microglia leading to the inflammatory response observed in PD.

There is conflicting evidence as to whether astrocytes are activated or not during the progression of AD and their role in disease pathogenesis. Some studies have reported the presence of activated astrocytes only in the initial stages of AD, which is followed by their inability to cope with the oxidative stress and inflammatory response, leading to the inactivation of astrocytes [14]. Other studies suggest there is a presence of activated astrocytes in certain regions of the brain when examining transgenic AD mice [15]. Under normal conditions, astrocytes play an important role in maintaining brain homeostasis and providing trophic support. We saw an increased activation of AD astrocytes in the treatment group and not in the control group showing that Ubisol-Q_{10} is able bring about changes in the morphology of astrocytes and that

astrocytes could play a role and facilitate the reduction of Aβ plaque deposits. Alternatively, it could be that the activation of the astrocytes is due to the resumption of basal levels of oxidative stress.

5.1 Conclusion & Overall Significance:

The work presented in this thesis showcases the importance of the successful development of preventative and/or therapeutic options for neurodegenerative diseases. This work establishes animal models that represent some of the clinical features of Parkinson's and Alzheimer's diseases. Importantly, I have been able to show that a water soluble formulation of Coenzyme Q10 effectively halts the progression of Parkinson's disease in an environmental toxin rat model. Furthermore, this formulation was not toxic and as such can be used long-term for the prevention of further neurodegeneration. These are important findings that provide a better understanding of the pathophysiology of AD and PD, and how mitochondrial dysfunction and oxidative stress contribute to the progression of these diseases. This better understanding is essential to developing more effective treatment options, as current treatment options only provide temporary symptomatic relief to the patients.

5.2 Future work:

The work presented in this thesis reveals more questions about the disease progression of AD and PD. Some of the work presented in this thesis is preliminary and as such, needs to be repeated and confirmed for conclusions to be made. Firstly, the protocols have to be standardised and the status of astrocytes and microglia has to be elucidated in the PD studies. Double staining could be carried out in order to see if the clumping of microglia is in fact around the Aβ plaque deposits. This would enable better understanding of the role of activated astrocytes in PD and how this can be exploited for therapeutic intervention. Immunohistochemistry with anti-nestin has to be standardized and staining could be carried out both separately and as a double stain along with anti-GFAP antibodies. This will help understand the reason behind PCNA positive immunostaining, in order to determine if the dividing cells belong to neuronal stem cells or if they are in fact the activated astrocytes.

Secondly, the AD animal study has to be repeated with more mice in each group and the mice have to be fed in individual cages in order to keep track of the water consumption. This way the water consumption by the mice will not be affected by dominance in the cage. Similar studies should be performed where the AD mice are provided with Ubisol-Q_{10} therapeutically, after the start of Aβ accumulation. The project should be further expanded and performed with other available transgenic mice models in order to gain a better understanding of the extent to which Ubisol-Q10 could provide relief if taken to clinical trials. Since senescence is one of the key features of AD, β-gal staining could be done and compared across the groups. Numerous studies indicate impaired fission and fusion and mitochondrial density along with bioenergetics at the synapsis. It would be interesting to perform immunohistochemistry to analyze the levels of mitochondrial fission and fusion proteins especially at the synapses where the number of

mitochondria is significantly higher. In addition, electron microscope images would aid in understanding the nature of mitochondria present in the neurons of both control and treatment mice. This would help gain knowledge on the effect this formulation has on the mitochondria, which houses the ETC and what we believe is the location where CoQ_{10} will be accumulated the most. AD is now being referred to as type 3 diabetes as impaired insulin signaling has been shown to play an important role in the pathogenesis of AD. Hence it would be interesting to study the phosphorylation status of the two proteins that play a role in the insulin signaling pathway, protein kinase B (Akt) and glycogen synthase kinase – 3β (GSK-3β), in control versus treatment groups. Type 2 diabetes models are being used to understand the link between diabetes and AD and to test various therapeutic options. Ubisol-Q_{10} could be tested in the type 2 diabetes animal models as well in addition to the transgenic mouse models of AD.

5.3 References:

1. Uttara, B., Singh, A. V, Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*, *7*(1), 65–74.
2. Duty, S., & Jenner, P. (2011). Animal models of Parkinson's disease: A source of novel treatments and clues to the cause of the disease. *British Journal of Pharmacology*, *164*(4), 1357–1391.
3. Cai, Q., & Tammineni, P. (2016). Alterations in Mitochondrial Quality Control in Alzheimer's Disease. *Frontiers in Cellular Neuroscience*, *10*(February), 24.
4. Spindler, M., Flint Beal, M., & Henchcliffe, C. (2009). Coenzyme Q10 effects in neurodegenerative disease. *Neuropsychiatric Disease and Treatment*, *5*(1), 597–610.
5. Cleren, C., Yang, L., Lorenzo, B., Calingasan, N. Y., Schomer, A., Sireci, A., … Beal, M. F. (2008). Therapeutic effects of coenzyme Q10 (CoQ10) and reduced CoQ10 in the MPTP model of Parkinsonism. *Journal of Neurochemistry*, *104*(6), 1613–1621.
6. McCarthy, S., Somayajulu, M., Sikorska, M., Borowy-Borowski, H., & Pandey, S. (2004). Paraquat induces oxidative stress and neuronal cell death; Neuroprotection by water-soluble Coenzyme Q10. *Toxicology and Applied Pharmacology*, *201*(1), 21–31.
7. Naderi, J., Somayajulu-Nitu, M., Mukerji, A., Sharda, P., Sikorska, M., Borowy-Borowski, H., … Pandey, S. (2006). Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis*, *11*(8), 1359–1369.
8. Houz, P., & Scherrmann, J. M. (1990). Toxicokinetics of paraquat in human. *Human & Experimental Toxicology*, *9*, 5-12.
9. Schierle, G. S., Hansson, O., Leist, M., Nicotera, P., Widner, H., & Brundin, P. (1999). Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nature Medicine*, *5*(1), 97–100.

10. D'Amelio, M., Cavallucci, V., & Cecconi, F. (2010). Neuronal caspase-3 signaling: not only cell death. *Cell Death and Differentiation*, *17*(7), 1104–14.
11. D'Amelio, M., Cavallucci, V., Middei, S., Marchetti, C., Pacioni, S., Ferri, A., … Cecconi, F. (2011). (Supplemental) Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. *Nature Neuroscience*, *14*(1), 69–76.
12. Xavier, A. L., Menezes, J. R. L., Goldman, S. a, & Nedergaard, M. (2014). Fine-tuning the central nervous system: microglial modelling of cells and synapses. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *369*(1654).
13. Stalder, M., Phinney, A., Probst, A., Sommer, B., Staufenbiel, M., & Jucker, M. (1999). Association of microglia with amyloid plaques in brains of APP23 transgenic mice. *The American Journal of Pathology*, *154*(6), 1673–1684.
14. Rodriguez-Vieitez, E., Saint-Aubert, L., Carter, S. F., Almkvist, O., Farid, K., Sch??ll, M., … Nordberg, A. (2016). Diverging longitudinal changes in astrocytosis and amyloid PET in autosomal dominant Alzheimer's disease. *Brain*, *139*(3), 922–936.
15. Matsuoka, Y., Picciano, M., Malester, B., LaFrancois, J., Zehr, C., Daeschner, J. M., … Duff, K. (2001). Inflammatory responses to amyloidosis in a transgenic mouse model of Alzheimer's disease. *The American Journal of Pathology*, *158*(4), 1345–54.

Appendix A

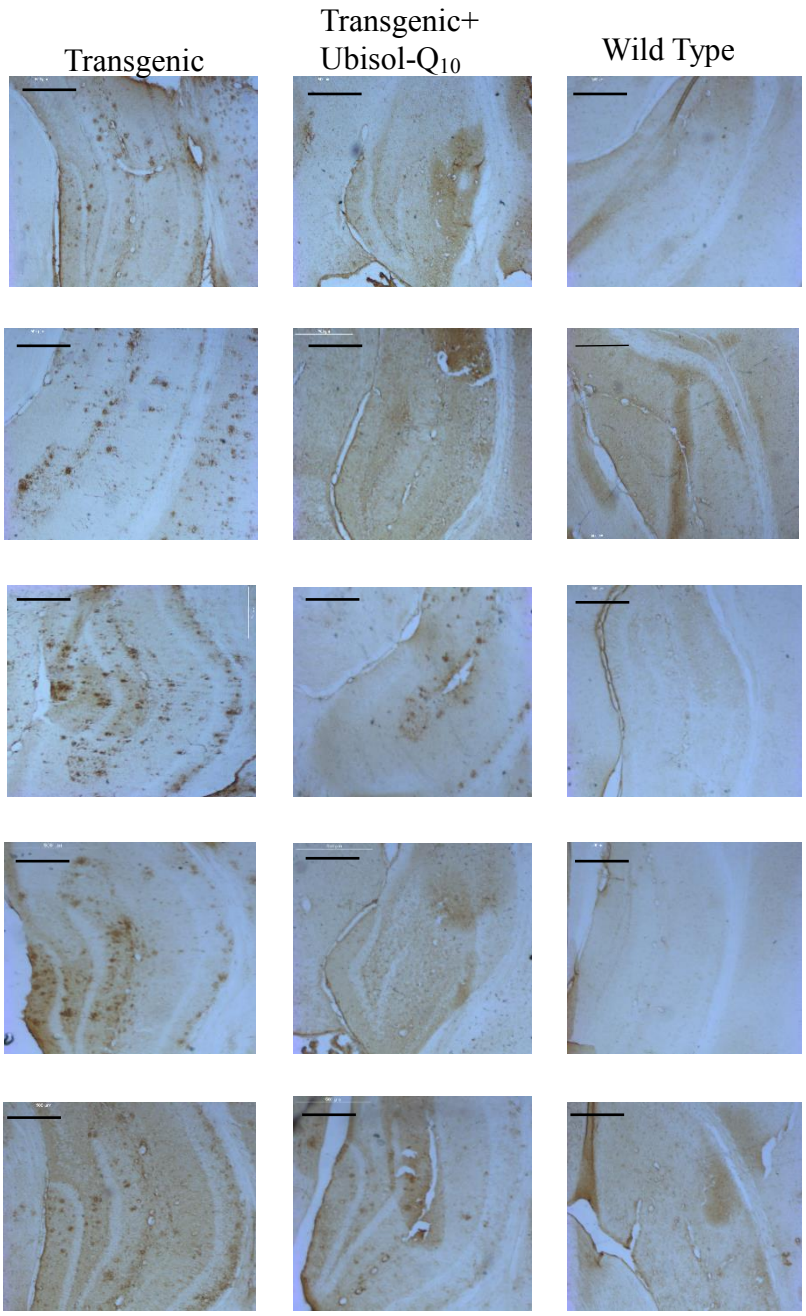


Figure 1. Immunohistochemistry with anti-amyloid-beta antibody to study the effects of prophylactic treatment with Ubisol-Q_{10} in an APP/PS1 transgenic AD mouse model. Each image represents a brain section from an individual mouse. There is greater levels of Aβ plaque deposit in the transgenic untreated group whereas three out of the five mice that received prophylactic treatment with Ubisol-Q_{10} showed near complete removal of plaque. Two mice showed a drastic decrease in the extent of plaque deposition. Wild type mice do not show any positive staining for amyloid plaque deposition. Scale bar = 500μm.

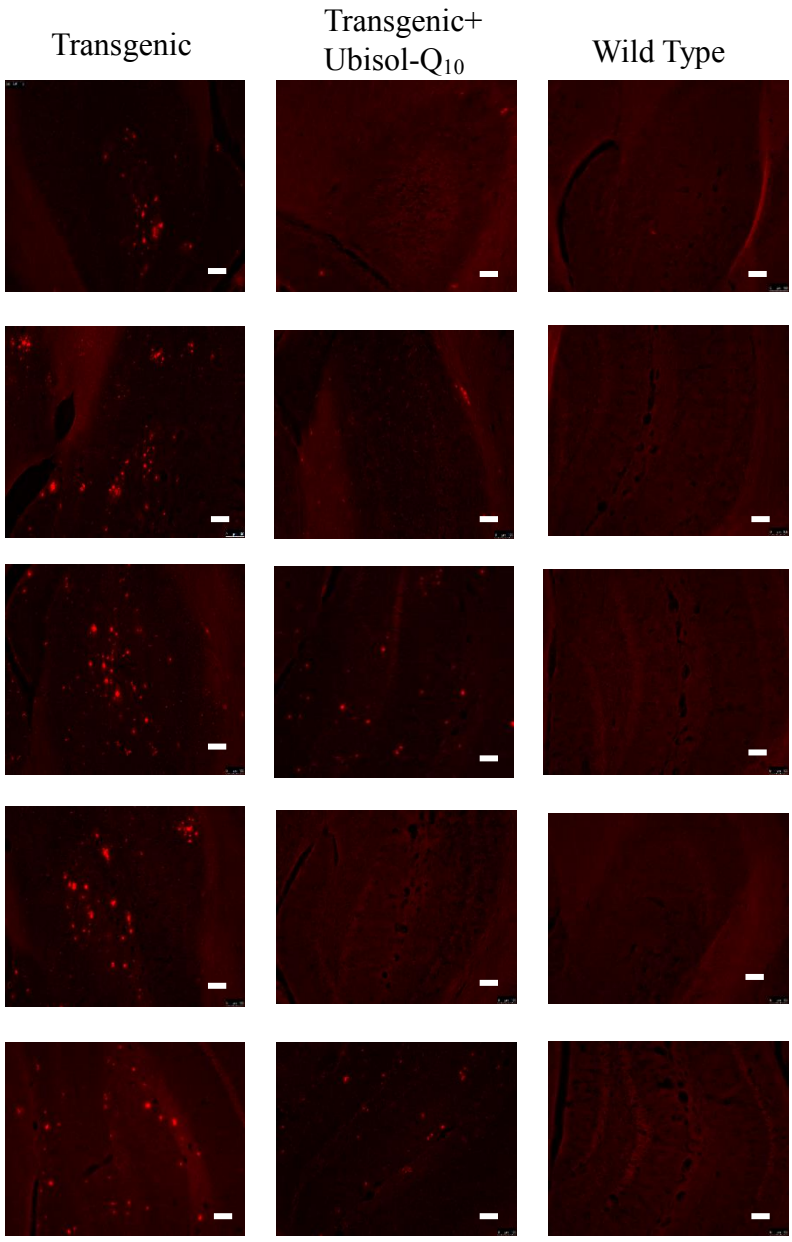


Figure 2. Congo red staining to study Aβ plaque deposition. Each image represents an individual mouse. Congo red staining was used to confirm anti-human Aβ antibody staining results. The same trend was observed where the untreated group has higher levels of plaque deposits while the Ubisol-Q_{10} treated transgenic mice demonstrate a near-complete or reduction in plaques. Wild type mice do not have plaque deposition. Scale bar = 100 µm

Appendix B

Table 1. Chemical reagents:

Chemical reagent	Company name	Catalogue number
Amyloid beta 40 ELISA kit	Thermo Fisher Scientific	KHB3482 kit
anti- human amyloid-beta antibody	Novus Biologicals	NBP2-13075
Anti-tyrosine hydroxylase antibody	Pelfreeze	P40101-0
DAB kit	Vector Laboratories	SK-4100
DAKO antibody diluent	DAKO	S0809
DAKO universal protein blocking solution	DAKO	X0909
rabbit polyclonal anti-glial fibrillary acidic protein antibody	Novus Biologicals	NB300-141
rabbit polyclonal anti-Iba-1 antibody	Novus Biologicals	NB100-1028
mouse monoclonal anti-neuronal nuclei antibody	EMD Millipore	MAB 377X
mouse monoclonal anti-proliferating cellular nuclear antigen antibody	DAKO	M087901-2
Vectastain Elite ABC HRP kit (Peroxidase mouse IgG)	Vector Laboratories	PK-6102
Vectastain Elite ABC HRP kit (Peroxidase rabbit IgG)	Vector Laboratories	PK-6101

Vita Auctoris

Name: Krithika Muthukumaran
Place Of Birth: Chennai, India
Year Of Birth: 1987
Education:
D.A.V Sr Secondary School, Chennai
2000-2002
D.A.V Senior Secondary School. Chennai
2002-2004
Ethiraj College for Women, Chennai
2004-2007
University of Leicester, Leicestershire